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CORRECTION

On page 454, Table I, Vol. 178, No. 1, March, 1949, in Column 5 read *1.71* for *1.37*, in Column 6 read *2.14* for *2.84*, and in Column 7 read *1439* for *1045*, *1541* for *1460*, and *80* for *40*.

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THE MOLECULAR MICRODISTILLATION OF CHOLESTEROL AND CHOLESTEROL ESTERS*

BY ALFRED E. KOEHLER AND ELSIE HILL

(From the Santa Barbara Cottage Hospital and the Sansum Clinic Research Foundation, Santa Barbara)

(Received for publication, November 4, 1948)

Fractional distillation of the sterols and their esters at the pressures ordinarily used requires temperatures that may result in deterioration. Hickman (1) and earlier workers have developed a cyclic molecular still which leaves little to be desired, except that it cannot be used for the extremely small amounts of lipides commonly encountered in blood and tissue analysis. We have devised a simple molecular still for such analysis, in which the distillation is made from a film and which is efficient quantitatively in amounts up to 5 mg. Not only has this still the advantage of using small amounts, but no vehicle is needed and so the distillate is recovered in pure form. The efficiency of the still is high. Hickman reports (1) that free sterols are removed at 180° and the esters at 250° in the cyclic still, whereas cholesterol in the film still is completely distilled at 88° and its esters at 155°. Though the film still is limited to relatively small amounts of material, it fulfils a definite need for analytical work.

Method

The still, as shown in Fig. 1, consists of two concentric tubes; the inner surface of the outer tube serves as the distilling surface and the inner tube as the condensing surface. The outer tube measures 200 mm. in length to the standard 24/40 ground joint and has an internal diameter of 25 mm. The inner, or condensing, tube has an outside diameter of 18 mm., giving a distance of 3.5 mm. at all points between the distilling and condensing surfaces. The still is connected through a large outlet at the top to a metal water-cooled fractionating oil diffusion pump of 20 liters per second capacity (Distillation Products VMF20). A Cenco megavac pump with a displacement of 31 liters of free air per minute was used for the fore pump. The high capacity of this system is of value in giving a uniform minimum pressure, not only throughout the distillation period but from day to day. The glass joint is sealed by means of a strip of adhesive tape around the outer joint. The tape projects $\frac{1}{4}$ inch upward and this well is filled with mercury. The smaller arm of the still is connected to a Pirani and a McLeod gage. The latter is used to check the Pirani gage frequently.

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* A preliminary report was presented at the Thirty-eighth annual meeting of the American Society of Biological Chemists at Chicago, May, 1947.

A glass jacket which fits on the rubber stopper is used for heating the outer tube. The jacket has a side arm at the top and bottom through which water is circulated at a constant temperature, or vapor is passed from a boiling liquid. A Wood's metal bath in a long steel tube with an electric heating coil on the outside is used for temperatures above 128° (amyl alcohol). This bath is raised so as to submerge the distilling tube when the desired temperature is reached and lowered again at the end of the distillation period.

In general the amount of lipide distilled has been between 1 and 2 mg. An aliquot of solution representing this amount of lipide is dried in the distilling tube, dissolved in 1 cc. of petroleum ether, and, by inclining the tube during rotation, dried in an even film up to three-fourths the length of the tube. The film covers an area of 95 sq. cm. and this represents about 0.015 mg. of lipide per sq. cm. An even film is important and can be obtained only if the surface is perfectly clean. Heating is started as soon as the pressure reaches 0 on the micron scale of the Pirani gage. This minimum pressure registers 0.003 ± 0.002 μ on the McLeod gage. Distillations are arbitrarily run for 30 minutes unless otherwise specified. At the end of the distillation period the oil diffusion pump is cooled as quickly as possible with an electric fan, air is admitted into the system, and the distilling tube removed. The condensing tube and the inner portion of the glass joint remain in a fixed position connected to the vacuum system and are not disturbed or disconnected. The condensate is washed down carefully with ether from the condenser tube into a beaker.

Analytical Data

Preparation of Material—Commercial spinal cord cholesterol was resaponified and crystallized six times from alcohol in dilute solution and had a melting point of 147.6°.

Serum cholesterol was prepared from mixed Bloor filtrates that had been stored in the refrigerator. The filtrates were concentrated at reduced pressure at 40° nearly to dryness and extracted with petroleum ether. After evaporation of the petroleum ether the lipides were dissolved in a small amount of warm 95 per cent ethyl alcohol and the sterol fraction separated by crystallization at 6°. The sterols were then recrystallized four times from 95 per cent alcohol to separate them from the other blood lipides further. The free cholesterol was separated from the esters by alumina adsorption according to the method of Trappe (2). Saponification was avoided because of the possible changes that might occur. This preparation still contained about 3 per cent of esters which were removed by distillation at 80°. The purified serum cholesterol had a melting point of 145.6° and probably still contained some contamination. The serum cholesterol esters were prepared from the lipides of the Bloor filtrates as described above. The free cholesterol was removed by precipitation with digitonin and the esters were crystallized at 6° from an ethanol solution of the lipides. No attempt was made to obtain the esters in a high state of purity, as repeated recrystallizations altered their composition. The cholesterol esters of the various fatty acids were prepared according to the method of Page and Rudy (3).

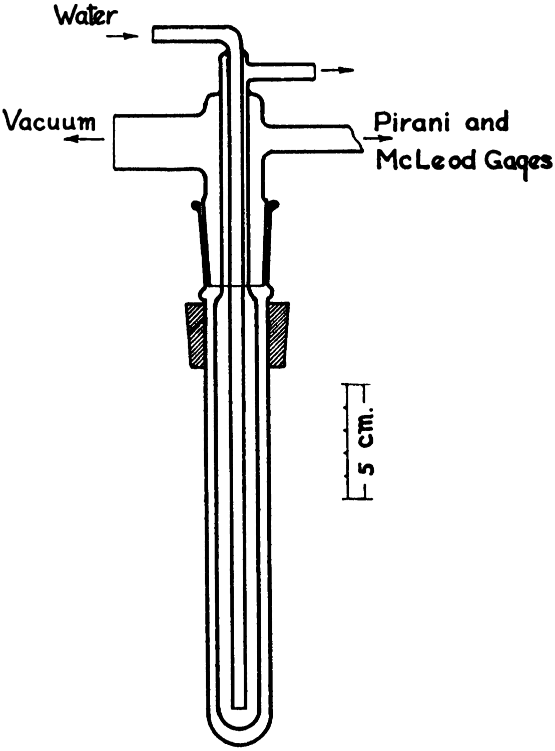

FIG. 1. Film type molecular still for distillation of small amounts of blood and tissue lipides.

Distillation of Cholesterol and Cholesterol Esters—The various preparations in amounts of 1.5 mg. were distilled for 30 minutes at differing temperatures. The cholesterol in the non-distilled portion, or residue, and in the distillate was determined by the Liebermann-Burchard color reaction.

The results of fractional distillation of cholesterol and its esters are shown in Fig. 2. Sublimation is appreciable at as low as 50°, and if heating were prolonged, all the cholesterol would volatilize at this temperature. However, at 88° all is sublimed in ½ hour. There is a sufficiently wide difference in volatility between the free cholesterol and cholesterol esters of the serum to permit a quantitative separation. Cholesterol palmitate, oleate, and stearate have distillation curves not greatly different from those of the serum esters, and a mixture of equal parts of these esters has a distilling characteristic approximately that of the serum esters. These esters, in addition to those of linoleic acid, were found as the main constituents of blood by Schoenheimer (4). In more recent and comprehensive work Kelsey and Longenecker (5) found that 85 per cent of the cholesterol esters of beef plasma were unsaturated and that linoleic acid comprised 82 per cent of the total. The distillation curves also show that none of the lower fatty acid homologues such as butyric acid are an appreciable part of the blood esters.

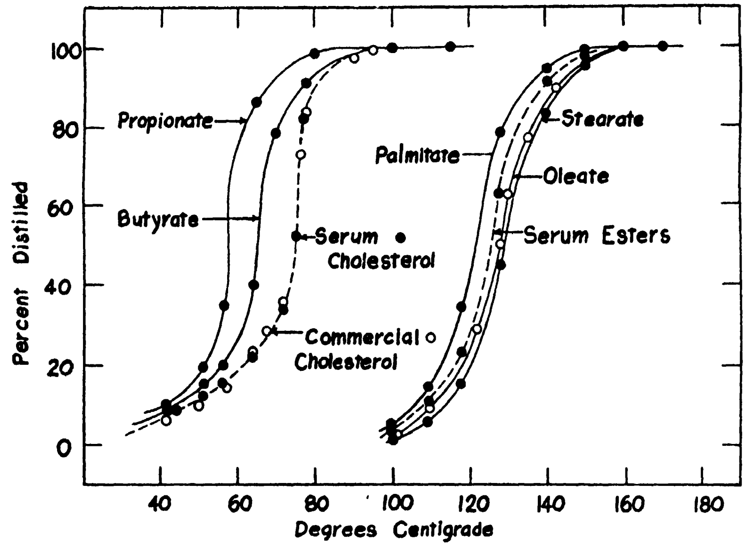

FIG. 2. Distillation curves for cholesterol and various cholesterol esters at 10^{-5} mm. of Hg pressure for 30 minutes.

Separation of Free Cholesterol from Its Esters—In the separation by molecular distillation the free cholesterol is available in an uncombined form, whereas in the digitonin separation it is regenerated only with difficulty. The application of this method of separation to the blood lipides is, of course, complicated by the effect of the other lipides on the rate and completeness of the cholesterol sublimation. Table I shows the effect of time of distillation on the amount of cholesterol distilled from purified cholesterol and also from dried Bloor filtrates. In both cases most of the cholesterol is distilled in 10 minutes, thus giving an ample margin for the 30 minute period. In Table II it is shown that the addition of sesame oil or cholesterol stearate does not appreciably affect the recovery of cholesterol. Under these conditions sesame oil is only partly distilled and no cholesterol stearate is distilled.

Comparison of Separation of Free Cholesterol in Serum Lipides by Distillation with Digitonin Precipitation—The total and ester cholesterols were determined in the sera of selected fasting subjects by means of digitonin precipitation according to the method of Bloor and Knudson (6). Aliquots of the alcoholic filtrate representing 1.5 mg. of total cholesterol were dried in the distilling tube under a water pump vacuum at room temperature and the lipides were deposited in an even film on the inner surface, as has been previously described. The distillations were then made for a period of 30 minutes at 88° or 100°. Table III shows a remarkable agreement between the results from the digitonin and the distillation methods in sera from healthy subjects. These subjects were selected to represent a wide range in serum total cholesterol, and although some values are outside the usually accepted normal range, careful physical and the usual laboratory examinations did not reveal any abnormalities. The values at both 88° and 100° indicate a considerable leeway in temperature for free cholesterol and ester separation. On the other hand, the free cholesterol percentage

TABLE I

Sublimation of Commercial and Serum Cholesterol at 88°

	Time of distillation	Residue	Distillate		Total recovered (residue + distillate)	
	min.	*mg.*	*mg.*	*per cent of total*	*mg.*	*per cent of original*
Cholesterol, 1.05 mg.	5	0.13	0.90	86	1.03	99
	10	0	1.02	97	1.02	97
	15	0	1.01	96	1.01	96
	30	0	1.02	97	1.02	97
Dried blood serum Filtrate 1, 10 cc.						
Total cholesterol, 0.80 mg.	5	0.61	0.20	25	0.81	101
Free " 0.19 " *	10	0.62	0.18	23	0.80	100
Ester " 0.61 "	30	0.60	0.18	23	0.78	97
Dried blood serum Filtrate 2, 10 cc.						
Total cholesterol, 0.84 mg.	5	0.68	0.14	17	0.82	98
Free " 0.18 " *	10	0.66	0.17	21	0.83	99
Ester " 0.66 "	30	0.62	0.19	23	0.81	96

* By digitonin precipitation.

of the total was 11 per cent lower at 88° and 6 per cent lower at 100° in the distillation method for the abnormal series. The explanation for this is not clear. In these sera there apparently is present a substance which is precipitated along with the free cholesterol by digitonin and gives the cholesterol color reaction, but is not distilled under conditions in which free cholesterol is readily and completely sublimed. This may be an altered form of cholesterol.

Distillation of Cholesterol Esters—Some insight into the composition of the cholesterol esters can be obtained by fractional distillation. Since nothing is known about the uniformity of the ester composition in blood or tissues in health or disease, an attempt has been made to see whether any gross differences exist. Bloor filtrates of various sera from fasting subjects were treated with digitonin to remove the free cholesterol. The ester fraction was distilled at 128° in amounts equivalent to approximately 1.5 mg. of cholesterol. The question naturally arose of the influence of variation in other lipides present on the amount of ester distilled. Table IV shows the effect of adding serum fatty acids and also of removing the phospholipides by acetone-$MgCl_2$ precipitation. The slight changes which occurred were probably within the range of experimental error. Furthermore, since a constant amount of ester cholesterol was always taken and since the neutral fats in general rise or fall with the cholesterol level, their relative ratios were probably little altered.

TABLE II

Recovery of Cholesterol from Added Lipides at 88°; Distillation 30 Minutes

	Residue	Distillate	Recovery of cholesterol
	mg.	*mg.*	*per cent*
0.5 mg. cholesterol + 1.0 mg. sesame oil	0	0.48	96
1.0 " " + 2.0 " " "	0	0.98	98
0.5 " " + 1.5 " cholesterol as cholesterol stearate	1.50	0.50	100
0.5 mg. cholesterol + 2.0 mg. cholesterol as cholesterol stearate	1.97	0.51	99

Table V shows the distillation of 1.5 mg. of ester cholesterol, expressed as cholesterol, from normal and abnormal fasting sera and of the intima of human aortas. The abnormal sera were from subjects with cirrhosis of the liver, diabetes, and hypertension. The conditions of distillation, namely time, temperature, and pressure, remained constant from one determination to the next. Although 128° falls on the steep portion of the ester curve as shown in Fig. 2, it was found that duplicate distillations gave comparable results. Furthermore, the standard deviation of the mean in sixteen different normal subjects as shown in Table V was relatively small for this type of analysis. The abnormal sera esters, however, were definitely less volatile and presumably contained esters of higher fatty acids. These findings merely indicate that such variations may exist in selected subjects, but not that they are characteristic of any particular disease.

TABLE III

Comparison of Separation of Free Cholesterol by Distillation and by Digitonin Precipitation; Distillation 30 Minutes

Subject No.	Diagnosis	Total cholesterol	Free cholesterol separation					
			By digitonin precipitation		By distillation			
					88°		100°	
		mg. per 100 cc.	*mg. per 100 cc.*	*per cent of total*	*mg. per 100 cc.*	*per cent of total*	*mg. per 100 cc.*	*per cent of total*
1. M. W.	Healthy subjects	590	175	30	148	25	143	24
2. I. B.		110	31	28	32	29	31	28
3. F. V.		163	38	23	44	27	46	28
4. H. W.		362	104	29	91	25	94	26
5. J. R.		226	63	28	61	27	59	26
6. C. F.		448	121	27	107	24	112	25
7. L. L.		332	80	24	90	27	80	24
8. M. S.		352	89	25	78	22	92	26
9. L. T.		236	41	17	47	20	45	19
Average		313		26		25		25
" of difference from digitonin method						−1		−1
Standard deviation of mean						±1.1		±1.0
1. G. T.	Cirrhosis of liver	290	117	61	154	53	162	56
2. W. W.	Schüller-Christian	527	242	46	221	42	232	44
3. E. M.	Diabetic ketosis	260	102	39	57	22	81	31
4. W. B.	" "	290	104	36	78	27	81	28
5. A. K.	Cirrhosis of liver	352	127	36	74	21	102	29
6. J. M.	" " "	262	94	36	68	26	81	31
7. D. M.	Diabetic fatty liver	275	96	35	69	25	66	24
8. E. H.	" " "	420	143	34	92	22	105	25
9. L. L.	Cardiac failure	220	73	33	46	21	55	25
Average		322		39		29		32
" of difference from digitonin method						−11		−6
Standard deviation of mean						±1.3		±0.9

The lipides of the intima and of the inner media of the aorta of adult subjects who had died from a variety of causes were extracted with warm

TABLE IV

Effect of Changes in Composition of Lipides on Cholesterol Ester Distillation at 128° for 30 Minutes

No. of subjects	Distilled esters		Difference	Standard deviation of mean
	per cent of total	*per cent of total*	*per cent*	
6	62	59*	−3	±2.6
10	62	63†	+1	±1.9

* 1 mg. of serum fatty acids added.
† Phospholipides removed.

TABLE V

Average Values for Distillation of Serum Cholesterol Esters at 128°; Distillation 30 Minutes

Esters	No. of subjects	Aliquot distilled, total ester cholesterol	Distilled esters		
		mg.	*mg.*	*per cent*	*standard deviation of mean*
Normal serum	16	0.76	0.55	72	±1.13
Abnormal serum	16	0.74	0.40	54	±2.50
Aorta	13	0.75	0.49	64	±2.23

TABLE VI

Distillation of Serum Cholesterol Esters in Diabetes at 128° for 30 Minutes

Subject	Uncontrolled			Controlled		
	Blood sugar	Esters	Distillate	Blood sugar	Esters	Distillate
	mg. per 100 cc.	*mg. 100 per cc.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
A. Z.*	410	235	55	98	185	70
R. S.	305	220	47	110	205	53
E. G.	298	225	67	114	185	72
E. D.	260	175	61	96	180	73
D. B.	380	355	72	76	210	71
L. B.	410	210	66	64	190	75
R. B.*	497	185	47	105	160	64
Average			59			68
Standard deviation of mean			±3			±2

* Coma.

alcohol in a homogenizer and the residue was further extracted with ether in a Soxhlet type of extractor. The alcohol and ether extracts were combined, dried *in vacuo*, dissolved in petroleum ether, and filtered. The free cholesterol was precipitated with digitonin. Aliquots representing 1.5 mg. of ester cholesterol were distilled.[1] The average yield of ester cholesterol from aorta was 56 per cent of the total cholesterol. The aorta esters were less volatile than the normal serum esters but were more volatile than the abnormal serum esters.

As is shown in Table VI, there probably is a shift in the nature of the esters in uncontrolled diabetes. The distilled portion in the controlled group was 15 per cent higher than in the uncontrolled group.

DISCUSSION

Molecular distillation from a thin film of lipides is of particular value in sterol separations because of the relatively low temperature required, the practically complete absence of oxygen, and the fact that the free cholesterol is uncombined and directly utilizable for further study.

The complete separation of free and ester cholesterol makes available another quantitative method besides that of digitonin precipitation. The fact that the two methods agree so closely for healthy subjects suggests that probably no other substance giving the Liebermann-Burchard color reaction enters into the free cholesterol fraction. On the other hand, in liver disease with low esters the free cholesterol percentage of the total runs 11 per cent lower with this method than with the digitonin method. Since the liver is instrumental in both the synthesis and disintegration of cholesterol, as well as in its esterification, it is quite possible that an abnormal liver metabolism might result in the formation of substances akin to but not true cholesterol.

The uniformity of normal serum cholesterol esters indicates that the nutritional status, variation in type of food ingested previous to fasting, differences in activity, and fluctuations in the total ester value have little if any effect on the ester composition. That this may vary, however, in conditions associated with various diseases, and that the shift is toward an increase in the higher fatty acid homologues are shown by our data. Since such a shift may indicate the presence of esters of a lower solubility in the blood serum, this could well be a factor in the deposition of cholesterol esters in the blood vessel walls. The finding that in uncontrolled diabetes the esters are also of the higher homologue type fits in with the fact that in diabetes there is a high incidence of arteriosclerosis.

A difficulty that might introduce an error in the ester fractionation studies is the fact that the esters are distilled from a rather complicated lipide mixture, variable constituents of which might influence the rate of distillation of the esters. In attempts to rule out this possibility, our efforts to separate the esters from the other lipides, particularly the neutral fats, by selective solubility, fractional crystallizations, and adsorption have always resulted in an alteration in the ester composition.

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[1] We are indebted to Dr. George Loquvam of the Department of Pathology for the dissection and preparation of the tissues.

SUMMARY

A molecular still of small dimensions and high efficiency is described for the analytical determination of cholesterol and its esters in blood and tissues.

The distillation curves of cholesterol and cholesterol esters from various sources are given. Cholesterol sublimes completely below 88° and can be separated quantitatively from the cholesterol esters of the blood lipides.

The percentage of free cholesterol in the total cholesterol of normal blood, as determined by distillation, agrees closely with values secured by the digitonin method, but results about 11 per cent lower are obtained in cases of liver disease with low ester value.

The fractional distillation of cholesterol esters indicates a fairly uniform composition in healthy subjects, but the esters associated with certain disease processes are less volatile. In uncontrolled diabetes the esters are less volatile than those in controlled diabetes.

The esters of the intima of human aortas are similar in composition to those of serum.

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THE DETERMINATION OF PROLINE IN PROTEIN HYDROLYSATES WITH LACTOBACILLUS BREVIS*

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Proline was first determined in 1901 by Fischer (2), who isolated copper L- and DL-proline dihydrates from an ester fraction of an acid hydrolysate of casein. During the ensuing three decades proline was determined essentially by this procedure in the hydrolysates of more than 50 proteins by Fischer (1901–11), Abderhalden (1902–36), Osborne (1906–11), and other workers. That data of satisfactory analytical value were probably unobtainable by the ester method was demonstrated by Osborne and Jones (3), who were able to recover only about 73 per cent of the proline from a mixture containing eleven pure amino acids. Because of the difficulties encountered in the ester fractionation method, proline has been determined by isolation as the hydantoin (4), cadmium chloride salt (5), picrate (6), rhodanilate (7), and the mercury salt of its N-methyl derivative (8) from butanol extracts, copper salts, or protein hydrolysate fractions of other types. Dakin (9) determined proline by extracting protein hydrolysates with butanol and crystallizing the proline from ethanol solutions which had been treated with mercuric acetate and barium hydroxide to precipitate other monoamino acids. Jones and Johns (10) determined proline by calculation from the total and amino nitrogen values of hydrolysates which had been freed from the basic amino acids by precipitation with phosphotungstic acid. Guest (11) proposed a method based on the colorimetric determination of pyrrole obtained by oxidation of proline; however, proline and hydroxyproline could not be differentiated by this procedure.

The solubility product principle was adapted by Bergmann and Stein (12, 13) to the quantitative determination of proline in gelatin hydrolysates. Although the method appears to yield reliable data when the numerous precautions emphasized by these authors are observed, it has not found wide application. Extensive data on the recovery of N-acetylproline from mixtures of acetylated amino acids have been reported by Synge *et al.* (14–16) and by Tristram (17). These authors stated that the proline values may be accurate to ±5 per cent. Polson *et al.* (18) have de-

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* Paper 53. For Paper 52, see Rockland and Dunn (1). This work was aided by grants from the Nutrition Foundation, Inc., the National Institutes of Health, United States Public Health Service, and the University of California.

TABLE I

*Composition of Basal Medium**

Constituent	Amount per liter	Constituent	Amount per liter
	gm.		gm.
Amino acids	15.8	KH_2PO_4	0.3
	mg.	Mineral salts†	0.24
DL-Alanine	920	$MgSO_4 \cdot 7H_2O$	0.12
L-Arginine HCl	520	$FeSO_4 \cdot 7H_2O$	0.006
Natural asparagine H_2O	1440	$MnSO_4 \cdot 4H_2O$	0.006
L-Cysteine HCl	600		mg.
L-Glutamic acid	1480	Vitamins‡	10.763
Glycine	260	Nicotinamide	9.0
L-Histidine $HCl \cdot H_2O$	260		γ
DL-Isoleucine	960	*p*-Aminobenzoic acid	1.5
L-Leucine	560	Biotin	1.5
DL-Lysine HCl	2680	Calcium *dl*-pantothenate	900
DL-Methionine	360	Choline chloride	120
DL-Norleucine	400	Folic acid	20
DL-Norvaline	400	Inositol	120
DL-Phenylalanine	560	Pyridoxal HCl	120
DL-Serine	500	Pyridoxamine 2HCl	120
DL-Threonine	1380	Pyridoxine HCl	120
DL-Tryptophan	160	Riboflavin	120
L-Tyrosine	480	Thiamine chloride HCl	120
DL-Valine	1880		mg.
	gm.	AGTU mixture§	120
Carbohydrates	52.5	Adenine	30
L-Arabinose	45.0	Guanine	30
D-Glucose, anhydrous	7.5	Thymine	30
Sodium acetate	45.0	Uracil	30
Phosphate buffer salts¶	0.6	HX mixture‖	60
K_2HPO_4	0.3	Hypoxanthine	30
		Xanthine	30

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* All the constituents were of C.P. quality. The medium is prepared conveniently as follows from the indicated quantities of constituents. Suspend the amino acids in 600 ml. of distilled water, add 10.0 ml. of the adenine-guanine-thymine-uracil solution, and stir the mixture until the amino acids dissolve. Add the glucose and sodium acetate and stir the suspension until the solids dissolve. Add 3.0 ml. of phosphate buffer salts solution, 3.0 ml. of mineral salts solution, 10.0 ml. of hypoxanthine-xanthine solution, 1.00 ml. of the vitamin solution, and the arabinose. Stir the mixture until the solid dissolves, adjust the pH of the solution to 6.8, and dilute the solution to 1 liter with distilled water. Steam, but *do not autoclave*, the final solution for 20 minutes at 100°. The concentration of the constituents of the basal medium in the final 3.0 ml. volumes of assay solutions was two-thirds that indicated in Table I.

† 0.1 N HCl solution containing 4.0 gm. of $MgSO_4 \cdot 7H_2O$, 0.200 gm. of $FeSO_4 \cdot 7H_2O$, and 0.200 gm. of $MnSO_4 \cdot 4H_2O$ per 100 ml.

TABLE I—*Concluded*

‡ 50 per cent ethanol solution containing 45.0 mg. of calcium *dl*-pantothenate, 450 mg. of nicotinamide, 1.00 mg. of folic acid, 0.075 mg. each of biotin and *p*-aminobenzoic acid, and 6.0 mg. each of the other vitamins listed per liter.

§ 0.5 N HCl solution containing 0.30 gm. each of adenine, guanine, thymine, and uracil.

‖ 0.5 N NaOH solution containing 0.30 gm. each of hypoxanthine and xanthine.

¶ Distilled water solution containing 10.0 gm. of K_2HPO_4 and 10.0 gm. of KH_2PO_4 per 100 ml.

scribed a method for the determination of proline by paper chromatography and ninhydrin analysis.

With the advent in 1943 of microbiological methods for the determination of amino acids it seemed a reasonable expectation that a satisfactory procedure could be developed for proline. That *Leuconostoc mesenteroides* P-60 might be employed for this purpose was indicated in 1944 by the report (19) that proline was essential for the growth of this organism. Two other lactic acid bacteria, *Lactobacillus arabinosus* 17-5 (20) and *Lactobacillus fermenti* 36 (21), have been shown not to require proline. Values have been reported, subsequently, for proline,[1] determined microbiologically with *Leuconostoc mesenteroides* P-60 and different basal media, including one described by the present authors (19). While the data obtained for proline in corn steep liquor (25), *botulinum* toxin (26), urine of mice (27) and humans (28), plant viruses (29), and purified proteins (30–34) appear to be satisfactory for comparative purposes, there is little basis, other than approximate agreement with literature values, on which to judge their probable accuracy.

Because of the observation (35) in 1947 that proline was synthesized by *Leuconostoc mesenteroides* P-60 after prolonged incubation on an enriched basal medium, it seemed probable that this amino acid could not be determined with high precision and accuracy with this organism and any available near optimal or suboptimal basal medium. It was conceivable that the incompleteness of the latter might be overcome by nutrients introduced into the assay solution by the test sample. In searching for a more promising organism a systematic study[2] was made of the nutritional requirements of five organisms which, as had been shown previously, synthesized proline only slowly even on an enriched basal medium (35) and produced a relatively large amount of acid on the same medium containing arabinose rather than glucose (36). The organisms investigated

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[1] Proline has been determined in several proteins by Brand *et al.* (22), who employed a *prolineless* mutant of *Neurospora crassa* and an unpublished method. A mutant strain of *Escherichia coli* which grows only in the presence of proline has been investigated by Tatum (23) and by Simmonds and Fruton (24).

[2] To be reported in a forthcoming paper.

TABLE II

Proline Found in Casein, Silk Fibroin, and Amino Acid Test Mixtures Simulating These Proteins[*]

Sample[†]	Per cent proline
Casein I[‡]	10.3 (3.1)
" II[§]	10.7 (2.2)
Silk Fibroin I[‖]	0.55 (1.0)
" " II[¶]	0.58 (1.2)
Casein I hydrolyzed[**] + 4% proline	101 (2.3)
" II " [**] + 4% "	98 (2.8)
" I + 4% proline, hydrolyzed[**]	103 (2.4)
" II + 4% " " [**]	98 (4.0)
Silk Fibroin II hydrolyzed[**] + 4% proline	96 (4.9)
" " II + 4% proline hydrolyzed[**]	104 (2.8)
Casein test mixture[††]	95 (2.7)
" " " [††] × 4	93 (2.1)
" " " [††] × 5	101 (1.5)
" " " [††] × 10	96 (1.6)
" " " [††] × 15	96 (1.2)
" " " [††] hydrolyzed[**] + 4% (total) proline	98 (1.6)
" " " [††] + 4% (total) proline, hydrolyzed[**]	95 (1.8)
Silk fibroin test mixture[‡‡]	99 (1.6)

[*] The purified sample of L-proline employed as the standard contained negligible moisture and ash. Its nitrogen content was 99.6 per cent of the theoretical amount and the specific rotation was $[\alpha]^{24.8}_{D} = -85.23°$ in water where $c = 1.4951$, $l = 4.0$, and $\alpha = -5.097°$. Another sample of L-proline, obtained through the courtesy of Dr. E. E. Howe (Merck and Company), was reported to be 99.5 per cent pure according to phase rule solubility determination. The specific rotation of this sample was found to be $[\alpha]^{24.8}_{D} = -84.79°$ and its activity towards *Lactobacillus brevis* 97.1 per cent that of the authors' preparation. Each value recorded for Casein I, Silk Fibroin I, casein test mixture × 4, and casein test mixture × 10 is a single determination. Each value recorded for Casein II, Silk Fibroin II, casein test mixture × 5, casein test mixture × 15, and silk fibroin test mixture is the average of two closely agreeing percentages found in separate experiments. Each value recorded for casein test mixture × 1, casein test mixture hydrolyzed plus proline, and casein test mixture plus proline hydrolyzed is the average of three closely agreeing percentages found in separate experiments. Each figure in the parentheses represents the mean deviation from the mean found for proline at the different levels of sample.

[†] D-Proline (6 to 34 γ) in DL-proline, hydroxy-L-proline (10 to 5000 γ), DL-ornithine HCl (10 to 5000 γ), and L-pyroglutamic acid (8 to 4000 γ) were inactive within detectable limits towards *Lactobacillus brevis*.

[‡] Described previously (38), containing 6.21 per cent moisture, 0.55 per cent ash, and 15.60 per cent nitrogen corrected for moisture and ash.

[§] Prepared essentially as described previously (38) and containing 9.66 per cent moisture, 0.63 per cent ash, and 15.35 per cent nitrogen corrected for moisture and ash.

[‖] Described previously (38), containing 5.68 per cent moisture, 0.25 per cent ash, and 18.7 per cent nitrogen corrected for moisture and ash.

TABLE II—*Concluded*

¶ Prepared essentially as described previously (38) and containing 1.12 per cent moisture, 0.0 per cent ash, and 17.95 per cent nitrogen corrected for moisture and ash.

** By heating approximately 1.0 gm. sample of protein or amino acid test mixture with 10 ml. of 5.97 N HCl for 18 hours at 120° in a Leiboff urea tube in an electrically heated Leiboff urea apparatus.

†† Composition essentially the same as that given previously (38).

‡‡ Composition essentially the same as that given previously (39), except for the addition of 2.8 per cent of L-aspartic acid, 0.40 per cent of L-tryptophan, and 6.8 per cent of DL-valine.

were *Lactobacillus lycopersici* (4005),[3] *Lactobacillus pentoaceticus* (367), *Leuconostoc mesenteroides* P-60 (8042), *Leuconostoc citrovorum* (8081), and *Lactobacillus brevis* (8257). A microbiological procedure for the determination of proline with *Lactobacillus brevis* and the arabinose-containing basal medium given in Table I is described in the present paper.

EXPERIMENTAL

The assay techniques were essentially the same as those described by Dunn *et al.* (37). The protein hydrolysates, the inoculum suspensions and solutions of the basal media, amino acid test mixtures, the standard amino acid and sodium chloride were delivered to 4 inch test-tubes with the aid of a Brewer automatic pipette (Baltimore Biological Laboratories). All solutions, except the basal media, were adjusted to the same concentration of sodium chloride to compensate for any stimulatory or inhibitory effects of this salt. The standard (fifteen levels), the amino acid test mixtures (ten levels), and the protein hydrolysates (ten levels) were each run in quadruplicate or triplicate.

All solutions (final volume, 3.0 ml. per 4 inch test-tube) were adjusted to pH 6.8 and sterilized by being steamed for 20 minutes at 100°. Solutions autoclaved for 10 minutes were colored deep orange. The inoculum was prepared by inoculating the previously described medium (37) with growth from a tomato juice-agar stab, incubating the inoculated solution for 24 hours at 35°, centrifuging the suspension, and washing the cells once with saline. Each assay tube was inoculated with 0.10 ml. of a suspension of the washed cells diluted with saline to an optical density equivalent to about 90 per cent transmission. The inoculated tubes were incubated for 72 hours at approximately 34°.

Satisfactory assay values were obtained over the range, 6 to 42 γ, of proline. At the 42 γ level of proline, acid was produced equivalent approximately to 19 ml. of 0.03 N NaOH (per 3.0 ml. final volume of solution per tube). Acid equal to about 1.7 ml. of 0.03 N NaOH was formed in each blank tube. Acid production in the standard and test sample tubes differed by 1.5 ± 0.9 per cent. The experimental results are shown in Table II.

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[3] American Type Culture Collection number.

DISCUSSION

The described assay procedure is considered to be satisfactory because acid production was relatively high, the standard curves were smooth and uniform except for a lag period up to 3 γ of proline, relatively little acid was formed in the blank tubes, and there was no evidence of any stimulatory or inhibitory effects. The recoveries of proline from amino acid test mixtures simulating the composition of casein and silk fibroin averaged 97 (93 to 102) per cent and the recovery of proline added to two different casein and two different silk fibroin samples, before and after hydrolysis of the protein or the mixtures, averaged 100 (96 to 104) per cent.

Some additional observations of particular significance were that the activity of L-proline was inappreciably altered by heating it for 18 hours at 120° with 6 N HCl in the presence and absence of other amino acids, and that hydroxy-L-proline, DL-ornithine, and L-pyroglutamic acid were inactive towards *Lactobacillus brevis.* The authors' method was found adaptable without significant decrease in precision or probable accuracy to assays of a protein (silk fibroin) and amino acid test mixtures with relatively low (about 0.5 per cent) proline content.

Proline in Casein

The proline found in casein (corrected for moisture and ash) averaged 10.5 (10.3 and 10.7) per cent for two samples of casein. This value is in good agreement with that (10.6 per cent) which Tristram[4] found by partition chromatography for a sample of casein containing 15.65 per cent nitrogen. This percentage of proline was considered to be reliable to ±2 per cent. Other values for proline in casein, both higher and lower than the percentage obtained in the present experiments, have been reported. These percentages include (*a*) 11.6 ± 0.7 (32), 11.2 (34), and 9.36 (31), determined microbiologically with *Leuconostoc mesenteroides* P-60, (*b*) 8.2 (11) and 8.1 (41) determined by colorimetric analysis of a degradation product (pyrrole), (*c*) 4.7 to about 10 (42–47), determined by calculation from the non-amino nitrogen of an ester fraction soluble in ethanol, (*d*) 6.7 (8) determined as the mercury double salt of stachydrin (dimethylbetaine of proline), and (*e*) 0.6 to 3.5 (1, 48–50) determined by isolation of the copper salt from an ester fraction.

[4] Quoted by Chibnall (40).

Proline in Silk Fibroin

The proline found in silk fibroin (corrected for moisture and ash) averaged 0.57 (0.55 and 0.58) per cent for two samples of silk fibroin. The proline content of silk fibroin prepared from silk proteins obtained from different sources has been found to vary from about 0.7 to 2.5 per cent by Abderhalden *et al.* (50–60) and other investigators (61, 62) on the basis of the copper salt isolated from ester fractions.

SUMMARY

A microbiological method has been described for the determination of proline in protein hydrolysates with *Lactobacillus brevis*. It was found that the proline content of moisture- and ash-free casein and silk fibroin was 10.5 and 0.57 per cent, respectively.

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THE RÔLE OF PTEROYLGLUTAMIC ACID IN TYROSINE OXIDATION BY RAT LIVER TISSUE

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In so far as their function in nutrition is defined, the vitamins have been found to be essential components of several enzyme systems (1). In this connection there is evidence that the vitamin, pteroylglutamic acid (PGA), is necessary in an enzyme system concerned with oxidation of the amino acid tyrosine. Experiments have shown that livers from rats with a PGA deficiency produced by incorporating succinylsulfathiazole in the diet have a lowered capacity to oxidize tyrosine (2), and that tyrosine metabolites disappear from the urine of vitamin C-deficient guinea pigs when PGA is administered (3). More indirect is the evidence that untreated pernicious anemia patients, who presumably are deficient in PGA (4), cannot properly oxidize tyrosine (5).

It is the purpose of this paper to present in detail our experiments on the oxidation of tyrosine by liver tissue from rats with a PGA deficiency induced by succinylsulfathiazole (2). It will be shown that the rate of tyrosine oxidation for these livers is lower than for normal liver and that the rate can be increased by the addition of PGA, but not by pteroylheptaglutamic acid, a naturally occurring conjugate of PGA, or by liver extract containing the antipernicious anemia (APA) principle.

These results will be compared and contrasted with those obtained in a new series of experiments in which the PGA inhibitor 4-aminopteroylglutamic acid is employed to produce a PGA deficiency state.

EXPERIMENTAL

Liver tissue was obtained from rats which had been maintained on the succinylsulfathiazole-containing diet of Daft and Sebrell (6) until leucopenia developed. This leucopenic state was taken as evidence that the animal was deficient in PGA. Further proof of the existence of a PGA deficiency was afforded by actual determination by a microbiological assay (7) of the PGA content of liver tissue from these animals. As is shown in Table I, the concentration of PGA in liver from rats receiving succinylsulfathiazole was much lower (less than 10 per cent) than that of liver from rats without this dietary supplement, when assayed either directly or after treatment under conditions simulating those in a Warburg experiment.

To determine the rate of tyrosine oxidation, livers from three to five animals were pooled and homogenized in a Waring blendor for 15 seconds, with M/15 phosphate buffer of pH 7.2 in an amount to give a concentration of 0.25 gm., wet weight, per ml. The oxidation of 0.5 gm. of L-tyrosine by 2 ml. of the suspensions was determined over a 2 hour period in the Warburg apparatus at 37°. In computing oxygen uptake, averages of three flasks were used. Nitrogen determinations on the different liver suspensions showed a relatively constant protein content per ml. To determine the fate of the tyrosine after incubation analysis for residual tyrosine was carried out on the pooled contents of the flasks used with each substance. The values so obtained agreed well with those calculated from the oxygen consumption. The oxygen uptake due to the utilization of the tyrosine was observed for normal and PGA-deficient liver tissue, and for PGA-deficient liver tissue to which PGA or PGA conjugate was added.

TABLE I

Pteroylglutamic Acid Content of Rat Liver Tissue

Rat group No.	Diet supplement	Preassay treatment of liver	PGA content of liver
			γ *per gm.*
1	None		4.5
1	"	2 hrs., 37°, pH 7.2	3.6
2	Succinylsulfathiazole		0.02
2	"	2 hrs., 37°, pH 7.2	0.22

A summary of the studies under these conditions (Table II) shows that in all of the experiments the oxidation of tyrosine by liver suspensions from the succinylsulfathiazole-induced PGA-deficient animals was less than that from normal rat livers. The addition of 10 γ of crystalline PGA per flask at least partially restored the oxidation. However, tyrosine oxidation was not affected by the conjugate, pteroylheptaglutamic acid, containing an equivalent amount of PGA.

Data in Table II also show the effect of liver extract containing the APA principle on the rate of tyrosine oxidation by PGA-deficient liver tissue. To the PGA-deficient homogenate 0.1 ml. per flask of phenol-free, 15 unit liver extract was added. No stimulation of tyrosine oxidation was noted, and in fact a decrease was observed with normal and PGA-deficient liver suspensions. Dilution of the liver extract to the point at which no inhibition occurred did not show any effect on tyrosine oxidation.

Recent experiments have reported the effects of a powerful antagonist of PGA, 4-amino-PGA, in producing a PGA deficiency syndrome in various animals including the rat (8, 9). It was of interest, therefore, to determine whether this inhibitor of PGA could affect the rate of tyrosine oxidation by liver tissue. Accordingly, 4-amino-PGA was added to normal liver suspensions and the tyrosine oxidation values were obtained as in the previous experiments. No effect on tyrosine oxidation was observed (Table III) when the antagonist was added in amounts up to 160 times (200 γ per ml.) the determined PGA content of liver (5 γ per gm.).

TABLE II

Tyrosine Oxidation by Liver Tissue from Rats with Succinylsulfathiazole-Induced PGA Deficiency

The results are expressed in c.mm. of O_2.

Experiment No.	Normal liver	PGA-deficient liver			
		Control	PGA added	PGA conjugate added	Liver extract added
1	66.5	17.5	23.0	18.1	
2	77.6	19.6	34.1	21.9	
3	48.6	19.3	26.8	17.2	
4	65.7	17.8	37.4	19.4	
5	67.0	32.3	47.3	34.8	
6	56.2	28.3	40.9	27.0	
7	48.5	19.3	26.8		13.3*
8	56.2	28.8	37.2		4.3*
9	64.7	27.1	42.6		
10	60.1	36.9	57.0		
11			34.0		7.4*
12			41.9		4.6*
13	63.0	47.4			48.3†
Mean......	61.3	26.8	37.4	23.0	

* 0.1 ml. of liver extract per flask.

† 0.0001 ml. of liver extract per flask.

It was found, however, that liver tissue from rats fed a diet containing 4-amino-PGA until a PGA deficiency syndrome developed was unable to oxidize tyrosine at a rate comparable to that of liver tissue from control rats (Table III). In contrast to results obtained with liver tissue from rats with a succinylsulfathiazole-induced PGA deficiency, the addition of PGA to liver tissue from inhibitor-fed rats in amounts up to 1 mg. per ml. of liver had no effect in restoring tyrosine oxidation. The effect of liver extract was also studied, and again no stimulation of tyrosine oxidation was noted with amounts of liver extract ranging from 0.0001 to 0.1 ml. per flask. Three experiments were carried out in which 1.04 γ of a vitamin B_{12} concentrate were added to flasks containing PGA-deficient liver homogenate, but no stimulation of tyrosine oxidation was observed under these conditions. However, liver tissue from rats receiving the same amount of inhibitor with a supplement of either PGA or liver extract in sufficient quantities to maintain a normal blood picture (8) had a tyrosine oxidation rate equivalent to normal liver controls. Since ascorbic acid has an effect on tyrosine oxidation in scorbutic guinea pigs (10), ascorbic acid was added to liver homogenates from both succinylsulfathiazole- and inhibitor-induced PGA-deficient animals. The addition of 0.25 mg. of ascorbic acid per flask had no effect on the tyrosine oxidation rate.

TABLE III

Tyrosine Oxidation by Liver Tissue from Rats with Inhibitor-Induced PGA Deficiency

The results are expressed in c.mm. of O_2.

Experiment No.	Normal liver		PGA-deficient liver				
	Control	Inhibitor added	Control	PGA added	Liver extract added	Vitamin B_{12} concentrate added	Treated *in vivo*
1	60.1		24.7	27.6 (10 γ)*			
2	42.4		28.0	20.6 (10 ")*			48.2†
3	67.8		29.3	25.3 (20 ")*		26.7	79.9†
4	63.0		48.5	47.8 (30 ")*	7.1 (0.1 ml.)‡	37.7	72.8§
5	56.4		36.5	32.0 (1 mg.)*	17.5 (0.01 ml.)‡		52.4§
6	64.7	61.8 (200 γ)‖	36.1		34.2 (0.0001 ")‡		
7	46.3	45.9 (250 ")‖					
8	61.2	66.8 (500 ")‖					
Mean..	57.7	58.1	33.8	30.6	19.6	32.2	63.5

* Amount of PGA per flask.

† PGA administered.

‡ Amount of liver extract per flask.

§ Liver extract administered.

‖ Amount of 4-amino-PGA per flask.

DISCUSSION

It has been shown that liver tissue homogenates from rats with a PGA deficiency syndrome induced by dietary supplements of either succinylsulfathiazole or a PGA inhibitor are unable to oxidize tyrosine at a normal rate. Further, the addition of PGA itself, under experimental conditions already described, increases the tyrosine oxidation rate of these liver homogenates. This is taken as evidence that PGA is an essential component of the tyrosine oxidative process.

In most of the experiments with succinylsulfathiazole-induced PGA-deficient liver tissue the addition of PGA *in vitro* effected only a partial restoration of the tyrosine oxidation rate, although a greater amount of PGA was added than is present in normal rat liver. It has been established that PGA is present in animal tissue predominantly as a conjugate (11), and the question therefore arises as to whether this conjugate is the metabolically active form of the vitamin. Since PGA conjugate (pteroylheptaglutamic acid), in contrast to PGA itself, had no effect on increasing the tyrosine oxidation rate of PGA-deficient liver tissue, it must be concluded that for its rôle in the tyrosine oxidative process, at least, PGA must function not in the conjugated but in the free form.

It has recently been shown (12) that a biotin antagonist could not displace the vitamin *in vitro*, but only affected the action of biotin added to biotin-deficient liver preparations. The finding that 4-amino-PGA will not affect the oxidation of tyrosine by rat liver tissue *in vitro* but that liver homogenates from 4-amino-PGA-fed rats are unable to oxidize tyrosine may be of similar significance in attempting to explain the mechanism of action of the antagonist. The results obtained with tyrosine oxidation of liver tissue from rats fed diets containing both 4-amino-PGA and PGA itself are proof that as far as tyrosine oxidation is concerned *in vivo* the action of 4-amino-PGA is completely reversed by PGA.

Reversal *in vivo* of 4-amino-PGA action was also obtained when liver extract was substituted for PGA. Hence, apparently there is a substance in liver extract that acts either directly or indirectly on the tyrosine oxidation process. Additional evidence for this is to be found in the recent report of Sealock and Lepow (13), wherein they show that APA liver extracts will return urinary tyrosyl values of scorbutic guinea pigs to normal levels. They were unable to demonstrate any effect on oxidation of tyrosine *in vitro* by liver slices from scorbutic guinea pigs. The experiments reported here are similar in that no effect on tyrosine metabolism was shown by either liver extract or vitamin B_{12} concentrate *in vitro*. Whether this substance in liver extract affecting tyrosine oxidation is actually the APA liver principle or vitamin B_{12} remains to be determined. Some evidence is afforded by experiments showing that patients with pernicious anemia excrete large amounts of tyrosine metabolites, a condition that is corrected by treatment with liver extract (5).

With scorbutic guinea pigs it has been found (13) that tyrosine oxidation is impaired, and that the addition of ascorbic acid will restore it to normal. Woodruff and Darby (3) reported that PGA would return urinary tyrosyl values to normal in scorbutic animals, and Johnson and Dana (14) showed that ascorbic acid produced improvement *in vivo* of a succinylsulfathiazole-induced PGA deficiency in rats. This improvement was not complete, and PGA was necessary for marked reticulocyte response. However, it has been shown here that the addition of ascorbic acid to the PGA-deficient liver suspensions had no effect on tyrosine oxidation under the conditions of these experiments.

SUMMARY

Liver tissue from rats with a succinylsulfathiazole-induced PGA deficiency shows a decreased oxidation of tyrosine compared with normal liver tissue. The oxidation of tyrosine can be partially restored by the addition *in vitro* of PGA, but not by PGA conjugate or by liver extract.

Liver tissue from rats with PGA deficiency induced by feeding 4-amino-PGA also shows a decreased tyrosine oxidation. This effect cannot be reversed by the addition *in vitro* of PGA, liver extract, or vitamin B_{12} concentrate but can be reversed by the administration *in vivo* of either PGA or liver extract. The addition of 4-amino-PGA *in vitro* to normal rat liver tissue does not affect the rate of tyrosine oxidation.

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THE ESTIMATION OF ANTIPYRINE IN BIOLOGICAL MATERIALS*

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A means of measuring the total water of the body *in vivo* has been developed (1). This method involves the use of antipyrine (1-phenyl-2,3-dimethylpyrazolone-5-one), the well known analgesic and antipyretic.

Two methods for the determination of this compound in biological materials are described in this report. The first, an extraction procedure, is suitable for its estimation in biological fluids and tissues. In this procedure antipyrine is extracted from the biological material with chloroform, the solvent evaporated, and the residue dissolved in dilute sulfuric acid. Sodium nitrite is added and the resulting 4-nitrosoantipyrine measured in a spectrophotometer at 350 mμ. This procedure, as indicated by a distribution technique (2, 3), possesses a high degree of specificity.

The second method, suitable for plasma, involves the estimation of the antipyrine directly in the plasma filtrate after deproteinization with zinc hydroxide. The speed and simplicity of this method recommend it for routine use. This procedure also has a high degree of specificity.

Extraction Procedure

Reagents—

1. Standard antipyrine solution, 100 mg. per liter. 100 mg. of antipyrine are dissolved in water and diluted to 1 liter. Working standards are made by diluting the stock solution with 0.07 N sulfuric acid.
2. Chloroform. A reagent grade of chloroform is washed successively with 1 N NaOH, 1 N HCl, and three times with water.
3. 1 N NaOH.
4. 0.07 N sulfuric acid.
5. 0.2 per cent sodium nitrite solution.

Procedure—Add 1 to 5 ml. of biological material[1] and 0.5 ml. of 1 N

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[1] Organ tissues and feces are prepared for analysis by emulsification in acid as described in a previous paper (2).

NaOH to a 60 ml. glass-stoppered bottle containing 20 ml. of chloroform. Shake for 10 minutes on a shaking apparatus. Transfer the contents of the bottle to a 30 ml. test-tube and centrifuge. Remove the aqueous layer by aspiration. Transfer 15 ml. of the solvent phase to a small beaker. Evaporate the chloroform to dryness at room temperature in a current of air. Dissolve the residue in 4 ml. of 0.07 N sulfuric acid. Transfer 3 ml. of the acid solution to a Corex or quartz cuvette and determine the optical density of the solution at 350 mμ in an ultraviolet spectrophotometer (Beckman). 0.07 N sulfuric acid is used for the zero setting. Add 2 drops of sodium nitrite solution to both the unknown and the zero setting, and read the optical density again after 20 minutes.[2] Subtract from this the optical density obtained before the addition of the nitrite.[3] Blank plasma, urine, or tissue run through the above procedure gives a negligible blank.

Precipitation Procedure

Reagents—

1. Zinc reagent. 100 gm. of $ZnSO_4 \cdot 7H_2O$ and 40 ml. of 6 N sulfuric acid are dissolved in water and diluted to 1 liter.
2. 0.75 N sodium hydroxide.
3. 4 N sulfuric acid.
4. 0.2 per cent sodium nitrite solution.

Procedure—To 2 ml. of plasma in a 50 ml. Erlenmeyer flask add 2 ml. of water and 2 ml. of zinc reagent. Add 2 ml. of 0.75 N sodium hydroxide drop by drop, with continuous swirling of the flask, and then shake for an additional half minute. After 10 minutes centrifuge at about 3500 R.P.M. for 15 minutes. Transfer 3 ml. of the clear supernatant fluid to a Corex or quartz cuvette, and add 1 drop of 4 N sulfuric acid. Read the optical density at 350 mμ. 3 ml. of water plus 1 drop of 4 N sulfuric acid are used for the zero setting. Add 2 drops of 0.2 per cent sodium nitrite solution to both the unknown and the zero setting and read the optical density again after 20 minutes.[2] Subtract from this the optical density obtained before the addition of the nitrite.[3] Normal plasma run through the procedure results in a small blank varying from 0 to 2 γ per ml. of plasma.

Standard Curve—Standards are prepared by placing 3 ml. of a known solution of antipyrine in 0.07 N sulfuric acid in a Corex or quartz cuvette and reading the optical density before and after the addition of sodium nitrite. 0.07 N sulfuric acid similarly treated is used as a zero setting. The optical densities are proportional to concentration. A concentration of 10 γ per ml. of antipyrine gives an optical density of about 0.350. Standards are run with each set of determinations, since there is a daily variation of about 3 per cent in the optical density.

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[2] The optical density reaches a maximum in about 20 minutes and fades slowly after about 35 minutes.

[3] The small optical density of the solution prior to the addition of the nitrite is not changed by the addition of nitrite and may therefore be corrected for by subtracting its reading from the final reading.

TABLE I

Recovery of Antipyrine Added to Plasma

Extraction procedure			Precipitation procedure		
Antipyrine added	Antipyrine recovered	Recovery	Antipyrine added	Antipyrine recovered	Recovery
γ	γ	*per cent*	*γ per ml. plasma*	*γ per ml. plasma*	*per cent*
10	10.3	103	12.5	12.3	98
	11.0	110		12.0	96
	10.1	101	20	20.0	100
	10.3	103		19.8	99
20	19.6	98		19.8	99
	19.8	99		20.0	100
	19.8	99		19.8	99
	19.8	99		20.0	100
	20.0	100	40	38.8	97
	20.0	100		39.6	99
30	29.1	97		39.6	99
	27.9	93		40.8	102
50	52.0	104		39.0	98
	51.0	102			
	48.0	96			
	48.0	96			

Results

Antipyrine added to plasma was recovered with adequate accuracy by both the extraction and precipitation procedures (Table I). Equally good results were obtained for other tissues with the extraction procedure. Analysis of plasma and urine stored in the refrigerator over a period of several days gave reproducible results, indicating that the compound is stable in biological fluids.

Specificity

It is important to know the identity of the substance measured, since the inclusion in the measurement of transformation products of antipyrine would invalidate the results.

The possible interference of metabolites of antipyrine was examined by the distribution technique (2, 3). The distributions of authentic antipyrine between ethylene dichloride and water at various pH values and between benzene and water at various pH values were compared with those of the apparent antipyrine extracted from plasma. This plasma was obtained from a human subject 5 hours after the oral administration of a 1 gm. dose. Within experimental error the distribution of the apparent antipyrine in plasma was the same as that of authentic antipyrine (Table II). It was concluded, therefore, that the material measured in plasma and presumably other tissues was actually antipyrine.

TABLE II

Distribution of Antipyrine and Apparent Antipyrine between Ethylene Dichloride and Water and Benzene and Water at Various pH Values

The apparent antipyrine was obtained by extraction with chloroform of the plasma of a subject who had received antipyrine. The chloroform solution was evaporated to dryness and the residue dissolved in water. Aliquots of this solution and of an authentic antipyrine solution were adjusted to various pH values and extracted with 2 volumes of benzene or ethylene dichloride. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to the total compound.

Solvent	pH	Authentic antipyrine	Apparent antipyrine from plasma
Ethylene dichloride	7	0.94	0.97
" "	1 (0.1 N HCl)	0.72	0.73
Benzene	7	0.81	0.79
"	3	0.61	0.58
"	1 (0.1 N HCl)	0.35	0.36

The plasma of subjects to whom antipyrine had been administered was analyzed by both the extraction and precipitation procedures. Both procedures yielded the same result, indicating that the precipitation procedure was as specific for plasma as was the extraction method.

The following drugs were tested for their interference in the procedure for antipyrine: phenacetin, aspirin, caffeine, codeine, morphine, atropine, sulfanilamide, sulfadiazine, phenobarbital, penicillin G, pentobarbital, acetanilide, pyramidon, demerol. With the exception of sulfadiazine, these drugs do not absorb light at 350 mμ, either before or after the addition of nitrous acid. Sulfadiazine interferes in the antipyrine procedure, since it not only absorbs light at 350 mμ, but this absorption is increased by the addition of nitrous acid.

SUMMARY

Two methods are described for the estimation of antipyrine in biological fluids and tissues. In the first, antipyrine is extracted from the biological material with chloroform, the solvent evaporated, and the residue dissolved in dilute sulfuric acid. Sodium nitrite is added and the resulting 4-nitrosoantipyrine measured spectrophotometrically.

The second method, suitable for plasma, permits the estimation of the antipyrine directly in plasma filtrate after deproteinization with zinc hydroxide.

The methods are specific for antipyrine in that they do not include any transformation products of the compound.

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THE USE OF ANTIPYRINE IN THE MEASUREMENT OF TOTAL BODY WATER IN MAN*

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Most methods for measuring the total water content of the body *in vivo* have been based on the degree of dilution of a foreign substance after its intravenous injection. These methods, recently reviewed by Pace (1), are in general unsatisfactory because the substances employed are not distributed evenly throughout the water of the body. Exceptions to this criticism are deuterium and tritium oxides (1–3), which presumably are distributed throughout the body like ordinary water. However, the costliness of these compounds and the complicated apparatus and techniques involved in their measurement make their use inconvenient. The proportion of water in the body may also be calculated from the subject's specific gravity (4), but this measurement is also laborious (5, 6) and there remains a need for a simple and accurate measure of body water.

For measurements of body water a substance should possess the following characteristics: (1) even and rapid distribution throughout body water; (2) non-toxicity in required doses; (3) slow transformation in, and slow excretion from, the body; (4) accurate and convenient estimation of its concentration in the plasma.

Prior studies in this laboratory on the distribution of antipyrine (1-phenyl-2,3-dimethylpyrazolone-5-one) in the dog indicated that it was distributed in tissues in proportion to their water content.[1] The possibility that antipyrine might be used in the measurement of total body water *in vivo* was therefore explored, and the results indicate that antipyrine fulfills the above requirements to a reasonably satisfactory degree.

Methods—Antipyrine in biological material was measured by a method previously reported (7). Deuterium oxide in plasma was measured by a modification of the method of Keston, Rittenberg, and Schoenheimer (8).

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[1] Brodie, B. B., and Axelrod, J., in preparation.

Results

Distribution in Body Tissues

The distribution of antipyrine was examined in representative tissues of two dogs given antipyrine intravenously. The tissues of one dog were examined 1½ hours and those of the other 2¾ hours after the injection. The animals were killed by an intravenous injection of air and the tissues sampled immediately. The tissue water was determined by drying to constant weight at 95–100°. The tissue concentration of antipyrine was measured and calculated in terms of tissue water. The antipyrine concentration in the water of various tissues was found to be nearly the same as that in plasma water, with the exception that the concentrations were somewhat low in the lung and somewhat high in the kidney (Table I). It appears that the concentration of antipyrine in tissues is determined chiefly by their water content.

TABLE I

Distribution of Antipyrine in Water of Dog Tissues

Dog 1 received 1.5 gm. and Dog 2 received 3 gm. of antipyrine intravenously. The tissues were examined 1½ and 2¾ hours, respectively, following injection.

Tissue	Antipyrine in tissue water		Ratio, $\frac{\text{tissue water antipyrine}}{\text{plasma water antipyrine}}$	
	Dog 1	Dog 2	Dog 1	Dog 2
	mg. per l.	*mg. per l.*		
Plasma...	29.2	54.6		
Heart....	31.1	56.8	1.07	1.04
Muscle 1.	30.6	51.0	1.05	0.94
“ 2..	30.7	54.6	1.05	1.00
Spleen....	30.1		1.03	
Kidney...	32.5	61.3	1.12	1.12
Liver.....	30.4	60.0	1.04	1.10
Lung......	24.3	47.1	0.83	0.86
Brain.............	28.9		0.99	
Cerebrospinal fluid..	25.7	53.8	0.88	0.99
Average...			1.01	1.01
Adipose tissue. Abdominal	26.3		0.90	
“ “ Groin.....	26.9		0.92	
“ “ Perirenal...	28.9		0.99	

The distribution of antipyrine in man was then studied. The tissues of two patients who were receiving antipyrine orally for several days prior to death were analyzed post mortem. Each had received the last dose of antipyrine about 5 hours before death. The tissue samples were removed and homogenized within 4 hours post mortem. A sample of blood was not obtained from the first patient. It is apparent that there is a relatively equal distribution of antipyrine in the water of the tissues, with the exception that the concentrations in the lung were somewhat low and in the liver somewhat high (Table II). Since the value for muscle in Subject 2 was low, additional specimens were obtained at surgical operation from two patients who had previously been given antipyrine. The ratios of muscle water antipyrine to plasma water antipyrine in these subjects were not low (1.04 and 1.06).

TABLE II

Distribution of Antipyrine in Water of Human Tissues

Both patients received antipyrine orally for several days prior to death and received their last dose about 5 hours before death. The tissues were removed for analysis within 4 hours post mortem.

Tissue	Antipyrine in tissue water		Ratio, $\frac{\text{tissue water antipyrine}}{\text{plasma water antipyrine}}$		
	Subject 1	Subject 2	Subject 2	Subject 3*	Subject 4*
	mg. per l.	*mg. per l.*			
Plasma		78.7			
Heart	88.0	78.0	0.99		
Kidney	87.0	78.5	1.00		
Liver	103.0	93.0	1.18		
Muscle 1	90.0	65.5	0.83	1.04	1.06
" 2	96.0				
Spleen	94.0	72.0	0.92		
Lung	88.0	70.5	0.90		
Skin	95.0	74.0	0.94		
Pericardial fluid		75.1	0.95		
Average			0.96		

* Specimens of muscle from Subjects 3 and 4 were obtained at surgical operation from two patients who had received antipyrine.

The antipyrine concentration in water of plasma and red blood cells was compared 1½ to 3 hours after the intravenous administration of 1.0 gm. doses of the drug to human subjects. The results indicate an approximately equal distribution between the water of red cells and plasma (Table III).

Patients with peripheral edema, ascites, and pleural effusion were given from 1.0 to 1.5 gm. of antipyrine intravenously. The concentration of antipyrine in the water of plasma and the particular fluid was compared

7 to 12 hours subsequently (Table IV). The drug appears to diffuse freely into abnormal depots of fluid.

The fact that most of the ratios of transudate to plasma were less than 1.0 suggested either incomplete distribution or some binding on plasma proteins. The extent to which antipyrine is bound to non-diffusible constituents of plasma was determined by dialysis against isotonic phosphate of pH 7.4 at 37°. The results indicated binding of about 10 per cent.

TABLE III

Distribution of Antipyrine between Human Red Blood Cells and Plasma

Twelve subjects were given 1 gm. of antipyrine intravenously, and the plasma and red cells compared 1½ to 3 hours later.

Sample No.	Antipyrine		Ratio, $\frac{\text{red cell water antipyrine}}{\text{plasma water antipyrine}}$
	Red cell water	Plasma water	
	mg. per l.	*mg. per l.*	
1	24.2	24.6	0.98
2	22.9	20.4	1.12
3	16.9	18.0	0.94
4	27.1	26.7	1.02
5	35.3	37.5	0.94
6	35.7	36.5	0.98
7	25.3	25.3	1.00
8	36.8	36.6	1.01
9	33.6	33.7	1.00
10	54.6	52.6	1.04
11	23.0	23.2	0.99
12	31.0	34.6	0.90

Excretion and Transformation

Urinary Excretion—The renal excretion of antipyrine was studied in eight human subjects after the intravenous administration of 1.0 gm. of the compound. Only 0.3 to 0.6 per cent of the amount injected was excreted in 4 hours following the injection. It is apparent that the excretion of the drug is negligible.

Transformation in Body—1 gm. of antipyrine was administered intravenously to 50 subjects, and plasma samples were taken at 2 hours and at various intervals subsequently. The results show that the rate of metabolism of antipyrine was on the average 6 per cent per hour, and varied in different subjects from 1 to 12 per cent. The rate was practically constant, however, over periods up to 12 hours in a given subject. It was therefore possible to correct for this chemical transformation by extrapolation of the logarithm of the plasma concentrations to zero time (Figs. 1 and 2).

The transformation of the compound in dogs was much more rapid than in humans, amounting to approximately 30 per cent per hour. For this reason the compound may be impractical for use in these animals.

TABLE IV

Distribution of Antipyrine between Plasma and Transudates

Subjects with peripheral edema, ascites, and pleural effusion were given 1 to 1.5 gm. of antipyrine intravenously and the plasma and transudate were obtained 7 to 12 hours subsequently.

Subject No.	Antipyrine	Ratio, $\frac{\text{transudate}}{\text{plasma}}$
	mg. per l. water	
1. Edema fluid	21.0	
Plasma	22.6	0.93
2. Ascitic fluid	26.4	
Plasma	28.6	0.92
3. Ascitic fluid	26.9	
Plasma	24.3	1.11
4. Ascitic fluid	24.7	
Plasma	26.0	0.95
5. Ascitic fluid	48.0	
Plasma	52.2	0.92
6. Pleural fluid	19.2	
Plasma	17.8	1.08
7. Ascitic fluid	22.7	
Plasma	25.6	0.89
8. Ascitic fluid	25.0	
Plasma	26.9	0.93

Time for Even Distribution of Antipyrine in Body

The logarithm of the plasma concentration was plotted against time for each of the above subjects (Fig. 1 shows typical results) and in all cases yielded a linear relationship, which indicates that the substance disappears at a rate proportional to its plasma concentration. In many cases 1 hour levels were also measured. These levels were sometimes a little above the straight line drawn through subsequent levels. The inference may be drawn that mixing for antipyrine in normals may not be quite complete in 1 hour, but is complete in 2 hours.

Antipyrine was administered to eight subjects with peripheral edema, ascites, or pleural effusion, and plasma samples taken at various intervals. Typical curves (Fig. 2 shows typical results) constructed as described for normal subjects indicate that the distribution of antipyrine in the body water of these subjects was complete but that a period of about 5 hours was required for uniform mixing.

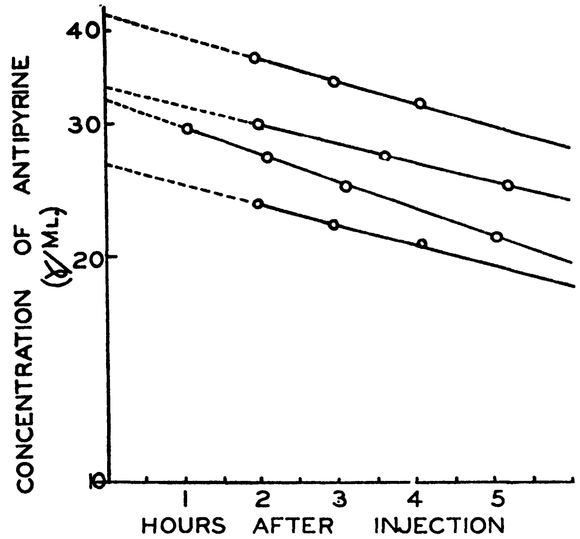

FIG. 1. Plasma levels of antipyrine in normal subjects at various intervals after intravenous injection. To correct for the metabolism of the drug during the time required for uniform distribution, the curve for the plasma level is extrapolated to zero time.

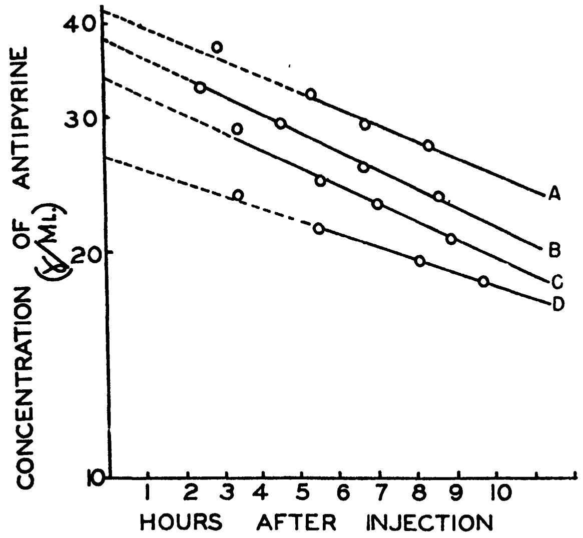

FIG. 2. Plasma levels of antipyrine in subjects with pathological fluid depots, at various intervals after intravenous injection. Subject A, congestive heart failure; Subjects B and C, nephritis; Subject D, cirrhosis. To correct for the metabolism of the drug during the time required for uniform distribution, the curve for the plasma level is extrapolated to zero time.

Toxicity

No untoward effects were observed in 200 patients given 1 gm. of antipyrine intravenously. Specifically, there was no methemoglobinemia or depression of the white blood cell count.

Measurement of Body Water with Antipyrine

The following technique is employed to measure body water. A control sample of blood is drawn, since there is a small blank value which must be used for correcting subsequent samples. 1 gm. of antipyrine in 50 ml. of water is injected intravenously from a burette or calibrated syringe. Blood samples are withdrawn at 2, 3, and 5 hours subsequently. All blood samples are heparinized. Plasma and cells are separated after centrifugation and the plasma stored in stoppered tubes for subsequent analysis of antipyrine.

The plasma concentration at zero time (the concentration at the time of injection if uniform distribution had been instantaneous and if none of the substance had been metabolized) is calculated by plotting the plasma levels on semilogarithmic paper and extrapolating the straight portion of the time-concentration curve (2nd, 3rd, and 5th hours) back to the time of injection.

The plasma water level of antipyrine is calculated by dividing the plasma level of antipyrine by the water content of the plasma.

The calculation for body water is made as follows:

$$\text{Body water (liters)} = \frac{\text{amount of drug injected (mg.)}}{\text{plasma water level (mg. per liter)}}$$

Comparison of Body Water Measurements Made with Deuterium Oxide and Antipyrine

To test the validity of antipyrine as a measure of body water, its volume of distribution was compared with that of deuterium oxide. This comparison was made in both normal subjects and subjects with abnormal depots of water.

Normal Subjects—1.0 gm. of antipyrine dissolved in 50 gm. of deuterium oxide was administered intravenously to eight normal subjects. Deuterium oxide and antipyrine in plasma were estimated at 2, 3, and 5 hours. The volume of distribution of deuterium oxide was taken as the average of 2, 3, and 5 hour values. The values for body water obtained by the two methods did not differ significantly, but those obtained with antipyrine were lower in most cases (Table V). The average difference was 1.2 liters and the maximum difference 3.1 liters. Body water as measured by antipyrine ranged from 39.3 to 57.9 per cent of the body weight, with an average of 51.9 per cent.

Abnormal Subjects—Seven patients, three with cirrhosis and ascites, two with nephritis and edema, and two in decompensated congestive failure with ascites and edema, were used as subjects. The antipyrine and heavy water in plasma were measured at 5, 7, and 9 hours following the administration of the two substances injected simultaneously. The measurements were made at longer intervals after injection than in the case of normal subjects, since the distribution of the compound takes longer when abnormal depots of fluid are present. The differences between the body water by the two methods were greater than in the case of the normals, averaging 2.7 liters, with a maximum difference of 5.1 liters (Table VI). In all cases the value obtained with antipyrine was somewhat lower. The body water as measured by antipyrine ranged from 51.7 to 70 per cent of body weight, with an average of 59.5 per cent.

TABLE V

Comparison of Total Body Water in Normal Subjects Measured by Antipyrine and Deuterium Oxide

1 gm. of antipyrine and 50 gm. of deuterium oxide were administered intravenously to each subject. Deuterium oxide and antipyrine were estimated in plasma 2, 3, and 5 hours subsequently and their volume of distribution calculated.

Subject No.	Sex	Weight	Total water				
			Antipyrine		Deuterium oxide		Deviation
		kg.	*liters*	*per cent body weight*	*liters*	*per cent body weight*	*liters*
1	F.	45.0	22.2	49.5	22.6	50.3	−0.4
2	"	73.4	28.8	39.3	30.8	42.0	−2.0
3	M.	66.5	34.2	51.5	35.8	53.9	−1.6
4	"	69.5	35.0	50.4	33.4	48.1	+1.6
5	"	70.0	39.9	57.0	39.8	56.9	+0.1
6	"	52.2	28.7	55.0	28.8	55.2	−0.1
7	"	55.5	30.5	55.0	33.6	60.6	−3.1
8	"	49.1	28.4	57.9	29.2	59.5	−0.8

The agreement between the results obtained with deuterium oxide and antipyrine is evidence that both are distributed in approximately the same volume of body water. Calculations for body water have been made without any attempt to correct for non-diffusible antipyrine. It may be that failure to do this introduces some error in the estimation of body water. This is suggested by the fact that the antipyrine space was usually somewhat smaller than the deuterium oxide space. However, this discrepancy may also be explained on the basis of exchange of some of the heavy hydrogen with readily exchangeable hydrogen in the body. It has been suggested that this exchange may account for several per cent of the total heavy hydrogen (3).

TABLE VI

Comparison of Total Body Water in Subjects with Abnormal Water Depots Measured by Antipyrine and Deuterium Oxide

1 gm. of antipyrine and 50 gm. of deuterium oxide were injected intravenously in each subject. Plasma deuterium oxide and antipyrine were estimated in 5, 7, and 9 hours and the volume of distribution calculated.

Subject No.	Sex	Diagnosis	Weight	Total water				
				Antipyrine		Deuterium oxide		Deviation
			kg.	*liters*	*per cent body weight*	*liters*	*per cent body weight*	*liters*
9	M.	Nephritis	49	31.0	63.4	32.0	65.4	−1.0
10	F.	Congestive heart failure	51.3	29.4	57.4	31.8	62.0	−2.4
11	"	Cirrhosis	55.6	30.3	54.5	31.2	56.1	−0.9
12	"	Nephritis	57.8	31.5	54.5	32.4	56.1	−0.9
13	M.	Cirrhosis	71.4	50.0	70.0	55.0	77.1	−5.0
14	"	"	74.1	38.3	51.7	41.8	56.4	−3.5
15	"	Congestive heart failure	82.2	53.5	65.0	58.6	71.3	−5.1

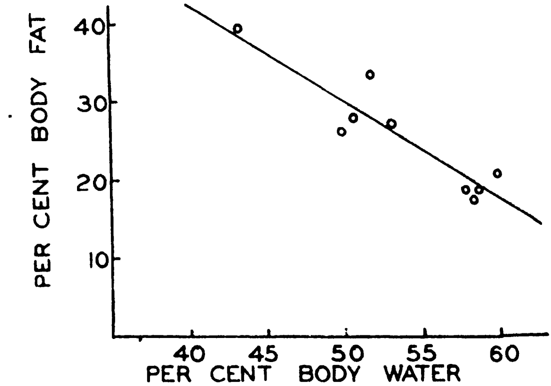

FIG. 3. Relationship of body water to body fat in human subjects

Relation of Total Body Water to Body Fat

Calculations of the body fat of subjects from the measurement of their specific gravity[2] have been carried out at the same time that total body water was measured by means of antipyrine. The results indicate that body water and body fat content bear an inverse relationship (Fig. 3).

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[2] Messinger, W., and Steele, J. M., in preparation.

Body water can also be calculated from the specific gravity (4). This calculation is based on the assumption that the total body water is 73 per cent of the fat-free body mass. Such calculations yield data for body water which agree fairly well with the antipyrine method (Table VII), a fact which constitutes a further and independent check on the validity of the antipyrine measurements.

TABLE VII

Comparison of Total Body Water in Normal Human Subjects Measured by Antipyrine and by Specific Gravity Methods

Subject No.	Per cent body water from antipyrine	Per cent body water from specific gravity
16	43.4	44.4
17	51.8	49.3
18	50.7	53.0
19	53.2	53.4
20	50.0	54.0
21	60.5	58.2
22	58.8	58.6
23	58.5	59.6
24	58.0	58.6

DISCUSSION

The few early figures for total water content of the body were provided by desiccation of post mortem tissues. The total body water was found to be 60 to 65 per cent of the body weight (9, 10). The generally accepted figure in modern text-books and monographs is roughly 65 to 70 per cent of body weight (11, 12).

The values obtained in the present study are lower than most current estimates and show considerable variability. In the nine normal individuals in whom both fat and water content of the body were measured, there was a clear inverse relationship between the amounts of fat and water, suggesting that the wide variability of body water is largely a function of the variability in body fat. The view that the diversity in body composition is largely a reflection of its fat content and that the composition of the residual lean body mass is fairly constant has been well documented for the body as a whole (4) and for individual tissues (13).

The figures for specific gravity, which Welham and Behnke give for a group of healthy men (14), indicate that a lean man contains 10 per cent fat, an average man about 20 per cent fat, and an obese one about 35 per cent fat. Body water in these individuals, calculated on the assumption that fat-free tissue is 73 per cent water, would be 66, 58, and 47 per cent water, respectively. The present figures of 50 to 60 per cent for normal males obtained by the use of deuterium oxide and antipyrine appear reasonable in the light of these estimates.

Antipyrine is not an ideal substance to use in the measurement of body water. It is bound by plasma protein to the extent of about 10 per cent and the magnitude of the error involved when no correction is made for protein binding is unknown. However, the good agreement between antipyrine and heavy water spaces indicates that the error is not large, and suggests that there is compensatory binding in the tissues. This is also indicated by the even distribution of antipyrine between plasma water and tissue water in dog and man.

A lesser disadvantage of antipyrine is its metabolism, which, although slow, requires the use of extrapolation through plasma levels at three concentrations to zero time. Search is proceeding for a compound that is not bound by plasma proteins and that is not metabolized in the body.

SUMMARY

The use of antipyrine in the measurement of body water in humans has been explored. This substance is uniformly distributed in the various tissues in close proportion to the water content, its excretion is negligible, and it is metabolized only slowly.

The volumes of distribution of deuterium oxide and antipyrine were compared in normal subjects and in subjects with abnormal depots of fluid. The values agree well. The volume of distribution of antipyrine also agrees well with the total body water as calculated from the specific gravity.

Ease of analysis and non-toxicity make antipyrine a satisfactory substance for use in the measurement of total body water, with minor qualifications in respect to plasma protein binding and slow metabolism.

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METABOLISM OF SYNTHETIC ESTROGENS. URINARY SULFUR PARTITIONING AFTER ESTROGEN ADMINISTRATION*

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In 1945 one of us reported in abstract (1) that little, if any, conjugation of diethylstilbestrol with sulfuric acid occurs in the rat and probably none at all in the rabbit. Since that time further interest in this subject has been evidenced by publications of experiments of the same type from other laboratories (see below). It seemed worth while, therefore, to extend our observations and publish them in some detail.

EXPERIMENTAL

Urinary sulfur partitioning was performed by Fiske's modification of Rosenheim's method (2). Sulfur excretion was determined for several 24 or 48 hour periods before and after the administration of a large dose of estrogen. Seven experiments were performed with rats, three with rabbits, three with cats, and two with dogs. In each case diethylstilbestrol was used, except for two rat experiments with "dienestrol" (4,4′-(diethylideneethylene)-diphenol), one rabbit experiment with "monomestrol" (the monomethyl ether of diethylstilbestrol), and one rabbit experiment with phenol. Injections were given intramuscularly in corn, sesame, or peanut oil. Sometimes the estrogen was given in acacia suspension by stomach tube. The animals were kept in metabolism cages, and rats, rabbits, and dogs were fed Purina chow. Cat 1 received meat and milk, and Cat 2 received milk alone. In some cases food intake was measured.

The results are summarized in Table I. It will be observed that the excess ethereal sulfate excretion after estrogen administration is quite variable. A definite increase occurred in two of the rat experiments

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(Rats 1 and 2, 3 and 4) with diethylstilbestrol, while in three the excess excretion was insignificant. No rise occurred after dienestrol in two rats. In the experiments on rats, the only definite changes were in a mixed strain of rats; those of a pure Long-Evans strain failed to show an increase.

TABLE I

Ethereal Sulfate Excretion during 4 Day Period Following Intramuscular Estrogen Administration

The excess of the preliminary average ethereal sulfate with the corresponding amount of estrogen this excess would represent if a monosulfate were formed.

Experiment	Estrogen	Ethereal sulfate excretion		Estrogen dose	Estrogen possibly excreted as sulfate	
		Preliminary average	Excess after estrogen			
		mg. S	*mg. S*	*mg.*	*mg.*	*per cent of dose*
Rats 1, 2*	Stilbestrol	2.4	16.5	750	138	18.4
“ 1, 2*	“ †	2.4	7.3‡	2000	61.0	3.1
Rats 3, 4*	“ †	4.3	31.5	1000	263	26.3
Rat 6*	“	1.1	2.2	450	18.4	4.1
“ 8	“	0.54	0	500	0	0
“ 9	Dienestrol	1.4	0.9	500	7.5	1.5
“ 10	“	0.76	1.4	500	11.6	2.3
Rabbit 3	Stilbestrol†	15.9	0	1500	0	0
“ 4	Monomestrol†	14.7	0	1770	0	0
“ 5	Stilbestrol	28.9	0	1000	0	0
“ 6	Phenol (control)	28.9	59.9	700	176	25.1
Cat 1	Stilbestrol†	32.6	0	2400	0	0
“ 1	“	32.6	0‡	1900	0	0
“ 2	“	11.2	12.8‡	1000	107	10.7
Dog 1	“	74.6	0	1000	0	0
“ 2	“	13.0	27.2	1000	227	22.7

* Mixed strain of rats; others are Long-Evans.

† Oral administration.

‡ Died within 2 days.

In rabbits no increase whatsoever in ethereal sulfate occurred after diethylstilbestrol in two experiments or after monomestrol in another. A small increase occurred after diethylstilbestrol in one cat, but as the cat died on the 2nd day, this may not be significant. A large increase was found in Dog 2 but none in Dog 1. A definite rise in ethereal sulfate, as was expected, occurred in Rabbit 6 which received phenol.

Since the ethereal sulfate excretion following estrogen administration was so variable, it is difficult to decide whether the rise that was occasionally seen is of any real significance; *i.e.*, does it indicate the excretion of a conjugate of the estrogen with sulfuric acid? In four cases out of twelve there was a definite excess ethereal sulfate excretion after diethylstilbestrol administration of over 10 per cent of the dose. This occurred in the mixed strain of rats, in one cat, and in one dog, but not in the Long-Evans rats or in the rabbits. Assuming complete and rapid absorption and the formation of a monosulfate of the estrogens, the amount of estrogen thus accounting for the excess sulfur and the percentage of the dose this represents can be calculated. These values are obviously rough approximations and represent the maximum amount of estrogen that might be excreted as a sulfate. It seems likely that conjugation of the estrogens with sulfuric acid probably does occur in these species except for the rabbit, but not to a great extent.

Neutral sulfur was usually depressed after estrogen administration. This would seem to eliminate the possibility of the formation of a mercapturic acid derivative of estrogen as a mode of detoxication.

Inorganic sulfate frequently fell markedly and was probably due to decreased food intake. Measurement of food intake in four cases showed changes parallel to those of inorganic sulfate.

DISCUSSION

Stroud (3) has isolated 14 per cent of an injected dose of diethylstilbestrol from rabbit urine as the free estrogen and 11 per cent after hydrolysis of a combined fraction. Mazur and Shorr (4) have isolated a monoglucuronide of diethylstilbestrol from rabbit urine following injection of the estrogen. They recovered 30 per cent of the administered dose in this form. Dodgson *et al.* (5) accounted for 71 per cent of diethylstilbestrol from rabbit urine as the glucuronide. They found no extra ethereal or neutral sulfur in rabbit urine after administration of this estrogen, dienestrol, or "hexestrol." They, as well as Simpson and Smith (6), have isolated glucuronides of the three estrogens from rabbit urine. In this paper (5) is presented a discussion of ethereal sulfate conjugation and it is remarked that the apparent absence of this conjugation in the case of synthetic estrogens is surprising. Smith and Williams (7) followed the excretion of free estrogens and their glucuronides by biological and gravimetric methods in rabbits after administration of the three above compounds. These workers found as much as 46 per cent of diethylstilbestrol was eliminated as a glucuronide. They were unable to isolate a sulfate, but stated they had indirect evidence of such conjugation of synthetic estrogen in man.

Since Zimmerberg (8) has shown that the amount of diethylstilbestrol conjugated by rat liver slices is decreased if $MgCl_2$ is substituted for $MgSO_4$ in the medium, it is presumed that this species has the ability to form a sulfate of the estrogen. Our data concur then with those of Dodgson *et al.* in that no evidence of sulfate conjugation was found in rabbits. We also agree with Zimmerberg in that the rat may conjugate diethylstilbestrol with sulfuric acid. In view of the high yields of glucuronide mentioned above and the low and variable ethereal sulfate excretions presented in this communication after estrogen administration, it would seem that sulfate conjugation does occur in some species, but it is probably not an important mode of detoxication of synthetic estrogens. From a consideration of the size of the estrogen dose in our experiments and the fact that deaths occurred so frequently, it would seem to be unprofitable to extend these studies further.

Our investigations are being continued in the direction of quantitative determinations of free estrogen, and combined estrogen, and glucuronic acid after administration of the synthetics.

SUMMARY

1. Urinary excretion of inorganic, ethereal, and neutral sulfur has been determined before and after administration of synthetic estrogens to rats, rabbits, cats, and dogs.

2. A rise in the ethereal sulfate fraction was sometimes found after diethylstilbestrol in rats, cats, and dogs, but not in rabbits. No increase was obtained with dienestrol in rats or with monomestrol in one rabbit.

3. The neutral sulfur fraction was usually decreased following estrogen administration. Inorganic sulfur usually fell markedly.

4. These results were interpreted to indicate that conjugation of diethylstilbestrol with sulfuric acid may take place in rats, cats, and dogs. The conjugation is variable in degree and at best involves only a small percentage of the dose. It fails to occur in rabbits. It also probably fails to occur with dienestrol in rats and with monomestrol in rabbits. Probably no mercapturic acid derivatives of any of the three estrogens are formed in the species studied. Changes in inorganic sulfate excretion are due to variation in food intake.

It is a pleasure to acknowledge the advice and encouragement of Dr. Ralph G. Smith of Tulane University.

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OLEIC ACID INTERFERENCE IN THE NEUROSPORA CRASSA ASSAY FOR BIOTIN

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A few years ago the author suggested that the *cholineless* mutant of *Neurospora crassa* might be used for the assay of biotin in milk products (1). At that time it was known that the microbiological response of *Neurospora crassa*, yeast, and the lactobacilli were not necessarily specific for biotin (1). Although the chemical structure of certain synthetic compounds such as desthiobiotin was known, the identity of certain naturally occurring interfering materials had not been identified. More recently it has been clearly demonstrated that, for a number of lactobacilli, biotin can be largely or completely replaced by a combination of oleic and aspartic acids (2–5). These findings bring up the question as to whether the biotin requirements of *Neurospora crassa* can also be met by a combination of oleic and aspartic acids. This report is an attempt to answer that question.

The investigations were made by the methods previously outlined (1). The work reported here does not include studies involving changes in the pH of the media or changes in incubation temperature. That such changes may affect the nutritive requirements of fungi has been discussed by Tatum (6). Since these conditions are controlled in the assay and are not, therefore, a source of error, they were not considered as variables in the present investigation.

Experiments designed to study the effect of oleic acid, aspartic acid, and Tween 80 (a polyoxyethylene derivative of sorbitan monooleate) have been carried out and are briefly summarized in Table I. Aspartic acid alone gives little stimulatory effect either in the presence or absence of biotin. Tween 80, which contains oleic acid in an esterified and readily dispersible form, shows minor growth activity in the absence of biotin and a slight stimulatory effect in the presence of biotin. Combinations of Tween 80 and aspartic acid are more active than is Tween 80 alone. In the presence of biotin, combinations of aspartic acid and Tween 80 show little or no greater activity than is shown by the latter compound.

Higher levels of Tween 80 and Tween 80 and aspartic acid than those reported in Table I were not tested. The more extensive data from which Table I was summarized suggested that at the higher level a plateau in the growth curve had been reached and that higher levels would not give appreciably greater growth.

TABLE I

Interference in Biotin Assays by Oleic and Aspartic Acids

Trial No.	Compounds added	Biotin per flask	Compounds added per flask	Dry mycelium
		mμgm.	*mg.*	*mg.*
1	None	0		2.2
	Biotin	1.0		27.7
	"	2.5		42.8
	Tween 80	0	0.8	5.2
	" 80	0	10.0	8.6
	Aspartic acid	0	0.8	2.9
	" "	0	10.0	2.3
	Tween 80 + aspartic acid	0	0.8 + 0.8	5.8
	" 80 + " "	0	10.0 + 10.0	15.4
	Biotin + Tween 80 + aspartic acid	1.0	0.4 + 0.4	27.2
	" + " 80 + " "	2.5	10.0 + 10.0	42.8
2	None	0		0.5
	Biotin	1.0		29.5
	"	2.5		46.1
	" in 10% acetone	1.0		22.1
	" " 10% "	2.5		44.7
	" " 10% alcohol	1.0		35.9
	" " 10% "	2.5		50.2
	" + Tween 80	1.0	4.0	31.0
	" + " 80	2.5	10.0	48.1
	" + " 80 + aspartic acid	1.0	4.0 + 4.0	31.5
	" + " 80 + " "	2.5	10.0 + 10.0	48.6
3	None	0		2.7
	Biotin	1.0		29.0
	"	2.5		47.1
	Oleic acid in 10% acetone		0.4	3.5
	" " " 10% "		1.0	5.6
	" " " 10% " + aspartic acid		0.4 + 0.4	3.5
	" " " 10% " + " "		1.0 + 1.0	2.9
	Biotin + oleic acid in 10% acetone	1.0	0.4	27.0
	" + " " " 10% " + aspartic acid	1.0	0.4 + 0.4	23.4

Oleic acid was first tested by dissolving in alcohol and dispersing the mixture in water. This mixture showed considerable stimulatory effect in the presence of biotin, but this stimulation was later discovered to be due to the ethyl alcohol rather than oleic acid. The stimulatory effect of alcohol has not yet been explained. The possibility that the effect is due to an impurity in the alcohol has not been entirely eliminated.

Acetone does not have the stimulatory effect given by alcohol and was, therefore, used in our later studies with oleic acid. The oleic acid is dissolved in acetone and diluted with this solvent so that 1 ml. of solution contains 20 mg. of oleic acid. 10 ml. of solution are then diluted with water to 100 ml. to give a suspension of oleic acid in 10 per cent acetone. This suspension was used directly or diluted further as required by the experiment being made. Free oleic acid shows only a minor activity. In the presence of biotin it may be slightly inhibitory.

Although the stimulatory effect of oleic acid and aspartic acid does not produce a large growth response of the *cholineless Neurospora crassa*, nevertheless an appreciable, if not serious, error, which is largely dependent upon the ratio of biotin to free and combined oleic acid, may be produced by combinations of these compounds in the microbiological assay for biotin with this organism. For precise work it may be necessary to extract the oleic acid from the sample with a fat solvent. This procedure has already been recommended for biotin assays with lactobacilli. Samples high in biotin or low in fat can probably be assayed without preliminary fat extraction by use of the *cholineless Neurospora crassa*, but it may be necessary to test each type of sample. Tests of this character have been carried out for whole milk powder and are summarized in Table II. These results demonstrate that a preliminary extraction of the samples with petroleum ether did not markedly affect the biotin assay results as determined with *cholineless Neurospora crassa*. Better agreement between the samples might be expected, but the variability falls within the range of ±20 per cent previously stated to occur with this organism (1). Although milk is rich in fat, it is also such a good source of biotin that only the equivalent of 4 mg. of milk solids is present in the assay flask at the highest level tested. Only 1 mg. of the 4 is fat, and only a fraction of milk fat consists of oleic and other un-

TABLE II

Effect of Petroleum Ether Extraction on Biotin Content of Dry Whole Milk Powder

Sample No.	Biotin per gm.	
	Original sample	Petroleum ether-extracted sample
	mμgm.	*mμgm.*
1	261	255
2	268	251
3	266	253
4	232	218
5	230	276
Average.........	251	251

saturateds fatty acid. Moreover, only a small fraction of the fat could be carried into the extract added to the flask when the samples are prepared according to the method outlined (1). For this reason one would scarcely expect that oleic acid would seriously interfere in the assay of milk for biotin with *Neurospora.*

SUMMARY

Under the conditions tested, oleic and aspartic acids will not completely replace biotin for the growth of the *cholineless Neurospora crassa.* For this organism, oleic acid and Tween 80 alone or in combination with aspartic acid give some growth response in the absence of biotin and a slight stimulatory response in the presence of biotin. Milk and probably other foods that are high in biotin may be assayed without preliminary fat extraction. Samples that are low in fat can also probably be assayed without fat extraction.

The author thanks the officials of the Pet Milk Company for releasing this report for publication. He especially thanks Dr. E. A. Louder, Technical Director, for his part in releasing the report, as well as for his supervision of the research program. The author acknowledges the laboratory assistance of Roberta Biggins.

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A PHOTOMETRIC METHOD FOR THE DETERMINATION OF α-AMYLASE IN BLOOD AND URINE, WITH USE OF THE STARCH-IODINE COLOR

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(Received for publication, December 20, 1948)

The use of the starch-iodine color for the estimation of α-amylase appears to be on a sound theoretical basis. Swanson (1) has shown that in the degradation of amylose by α-amylase there is a random attack by the enzyme upon the polysaccharide chain yielding hexose units of varying lengths. Swanson (2) has further observed that chains 4 to 6 glucose units in length give no color with iodine, chains containing 8 to 12 units give a red color, and chains of 30 glucose units, or longer, yield a blue color.

We have developed a starch-iodine method for the determination of α-amylase in serum and urine in which the blue color formed by the reaction of starch with iodine is measured photometrically before and after incubation of soluble starch with material containing the enzyme. The decrease in blue color obtained after the incubation is a measure of the amylase concentration. When appropriate conditions are set up, starch-iodine color values are obtained that are proportional to the amount of enzyme present and to the time of incubation, with use of a fairly wide range of concentration of substrate.

The use of the photoelectric colorimeter removes the limitations inherent in starch-iodine methods in which an end-point is visually selected. In addition to being sound theoretically, the proposed method has certain practical advantages: It requires less work and time than the saccharogenic methods, the procedure is simple and is applicable to the estimation of amylase in blood and urine without change in the basic technique, and it permits the achievement of a high degree of accuracy and precision.

Method

Reagents—

1. Substrate. A 1.2 per cent solution of soluble starch is made up at the time of use. Weigh accurately 1.2 gm. of Merck's soluble starch (Lintner). Suspend this in about 10 cc. of distilled water in a 100 cc. volumetric flask. Make up to slightly under volume with boiling dis-

tilled water. Place the flask in a boiling water bath for 3 minutes and make up to volume. Allow the starch and bath to cool to 90° and hold at that temperature during pipetting.

2. Phosphate buffer, pH 7.2 (Myers, Free, and Rosinski (3)). Dissolve 7.62 gm. of anhydrous potassium dihydrogen phosphate and 20.45 gm. of disodium hydrogen phosphate in distilled water in a liter flask and make up to volume.

3. 0.5 M sodium chloride.

4. N hydrochloric acid.

5. Iodine reagent. Dissolve 30 gm. of potassium iodide and 3 gm. of iodine in distilled water in a liter flask and make up to volume.

Procedure

Pipette 5 cc. of 1.2 per cent starch solution (60 mg.) at approximately 90°, 3 cc. of phosphate buffer, and 1 cc. of 0.5 M sodium chloride into each of two test-tubes, one labeled A for the digest, and one labeled B for the control with undigested starch. Into a third tube (C), the blank, pipette 5 cc. of distilled water, 3 cc. of phosphate buffer, and 1 cc. of 0.5 M sodium chloride. Place all tubes in a water bath at 37° until they have reached the temperature of the water bath. To Tube A (for the digest) add 1 cc. of enzyme solution (serum, plasma, or urine). Keep all tubes in the water bath for exactly 30 minutes. Promptly add 2 cc. of N hydrochloric acid to each tube. This brings the pH below 2, a step that stops amylase action in the digest tube and prevents action of the enzyme next added to the control tube. Add 1 cc. of enzyme solution to Tubes B (control) and C (blank) and mix thoroughly. Pipette 2 cc. of each of these reaction mixtures into appropriately labeled 500 cc. volumetric flasks containing about 400 cc. of distilled water and 5 cc. of N hydrochloric acid. Add 1 cc. of iodine reagent to each flask and make up to volume. The resulting blue solutions are decanted into cuvettes and read in a photoelectric colorimeter at a wave-length of 620 mμ. The colorimeter is set at 100, or the null point, with solution from Tube C. The latter usually reads 99.75 on the Evelyn colorimeter against distilled water; hence distilled water may be used for the colorimeter setting without materially affecting the results. Solution from Tube B gives the iodine color value without amylase action and solution from Tube A gives the value after enzyme action.

Calculations—Let $D = 2 - \log G$ = optical density

$$\frac{(D \text{ of control}) - (D \text{ of digest})}{(D \text{ of control})} \times 60 = \text{mg. of starch hydrolyzed}$$

The amylase unit is defined as the amount of enzyme that under the conditions of this procedure, with 60 mg. of starch present, will hydrolyze 10 mg. of starch in 30 minutes to a stage at which no color is given with iodine at 620 mμ. The definition of this amylase unit was established to make the unit conform as closely as possible to the units of methods in general use.

For plasma or serum the calculation is

$$\frac{(D \text{ of control}) - (D \text{ of digest})}{(D \text{ of control})} \times \frac{60}{10} \times 100 = \text{amylase units per 100 cc.}$$

DISCUSSION

As shown in Fig. 1, the blue color developed by this procedure has been found to conform to the Beer-Lambert law throughout the range of concentration of starch used. The region of maximum absorption of the color was found to be between 600 and 620 mμ (Fig. 2, Curve B). We have adopted 620 mμ as the most desirable wave-length for readings for several reasons: The absorption due to the iodine reagent alone is negligible at 620 mμ (Fig. 2, Curve C); Hanes and Cattle (4) and Swanson (1) have shown that as α-amylase hydrolyzes amylose, producing shorter chains of hexose units, the range of maximum absorption of the starch-iodine color shifts towards the lower wave-lengths. Our observations are in accord with the work of these authors, as shown by the absorption curve for starch hydrolyzed by amylase (Fig. 2, Curve A). The use of the higher wave-length reduces to a minimum the possibility of interference by the short chain products of hydrolysis.

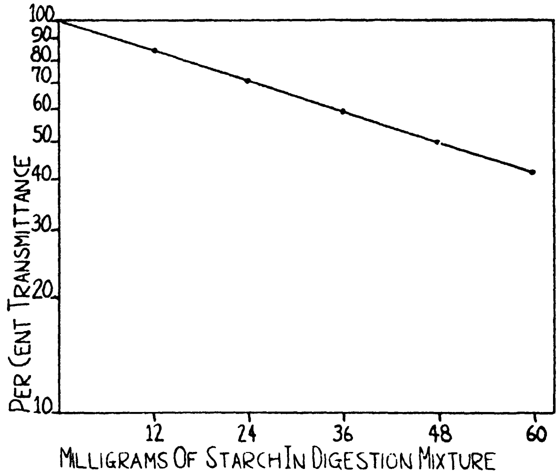

FIG. 1. The relation of the starch-iodine color intensity to the concentration of starch.

Starch and iodine concentrations were selected to give a range and flexibility well suited to clinical use. The technique outlined permits the accurate determination of amylase concentrations in the blood up to 500 units per 100 cc. For greater concentrations of enzyme the serum should be diluted. Marked changes in temperature are known to affect the intensity of the starch-iodine color. At room temperatures, however, the color produced is stable.

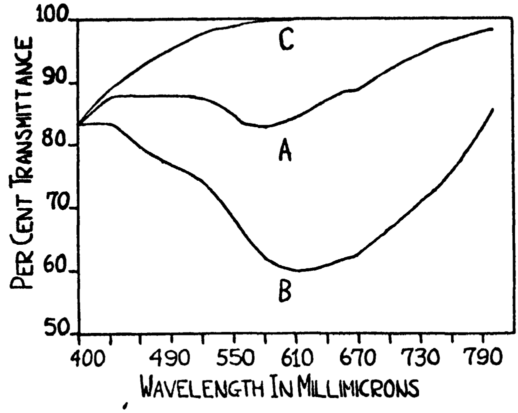

FIG. 2. The absorption curves of the colors used. Curves A and B, the color obtained by treatment of hydrolyzed and unhydrolyzed soluble starch, respectively, with iodine reagent; Curve C, the iodine reagent only.

TABLE I

Comparison of Ten Starches with Use of Urine As Source of Enzyme

With the exception of the first two samples all starches were made soluble by the method of Small (5). The amylase values are averages of three determinations.

Starch	Amylase
	units per 100 cc.
Merck soluble, Lot 42196	183.2
" " " 42477	198.0
Potato, laboratory Preparation 1	214.2
" " " 2	196.4
Corn, commercial, extracted 48 hrs. with dioxane	162.0
" not extracted	150.7
Rice*	109.6
Sago palm*	110.2
Sweet potato, laboratory preparation	153.0
Repetition of 1st starch after 48 hrs.	178.8
Potato, Merck	165.4

* Kindly furnished to us by Dr. C. S. Hudson of the National Institutes of Health.

The starch used in these experiments was Merck's soluble starch

(Lintner). This starch was chosen because it is readily available commercially and requires no special treatment before use. It was expected that starches from different sources would not give the same results with this procedure due to variations in composition. A series of determinations was made upon ten starches of different types and also upon different preparations of the same starch. The enzyme solution used was human urine. Table I shows the results of these experiments. The first four starches tested, which were of Irish potato origin, gave comparable results. Corn-starch gave higher values after extraction with dioxane. Irish potato starch gave the highest values of all starches examined. The last sample of starch in Table I was raw starch from a commercial source which was made soluble by the method of Small (5). No explanation is obvious for the low value obtained with this starch as compared with other Irish potato starches.

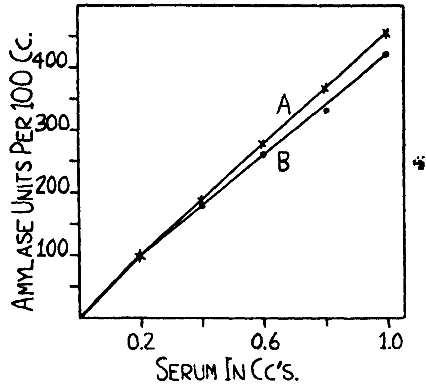

Fig. 3

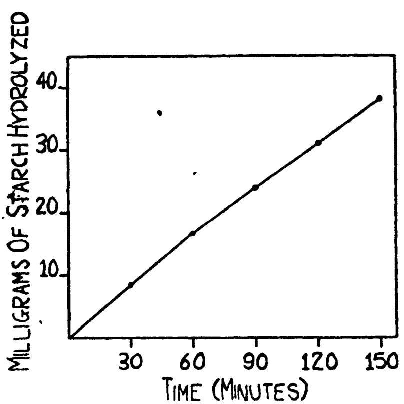

Fig. 4

Fig. 3. Curves showing amylase activity of serial dilutions of serum with (Curve A) phosphate buffer and (Curve B) HCl-NaCl buffer.

Fig. 4. The relation of α-amylase activity to the time of incubation, with use of 1 cc. of human serum as the source of enzyme.

We have adopted the phosphate buffer used by Myers, Free, and Rosinski (3). We found this buffer satisfactory for serum and urine. The HCl-NaCl buffer used by Somogyi (6) appears adequate for serum in lower ranges of values but at high enzyme concentrations the phosphate buffer gave higher values, as shown in Fig. 3. The lower activity observed with the HCl-NaCl buffer is more marked in urine.

A series of experiments was performed to study further the validity of the procedure. In the first experiment rabbit serum was used as the enzyme source. The amount of enzyme present was held constant and the starch content of the digest varied from 12 to 120 mg. The results

TABLE II

Effect of Variation of Starch Concentration on Enzyme Hydrolysis

Amount of starch in digest	Amylase
mg.	*units per 100 cc.*
12	83.2
24	83.7
48	88.8
72	82.6
120	86.8

TABLE III

Effect of Serial Dilution of Enzyme on Hydrolysis of Starch

60 mg. of starch were used in each tube.

Enzyme solution	Urine amylase	Serum amylase
cc.	*units per 100 cc.*	*units per 100 cc.*
0.2	73.2	106.9
0.4	142.8	198.5
0.6	218.0	303.6
0.8	288.6	400.0
1.0	377.4	478.8

TABLE IV

Amylase Values of Fifteen Human Sera Determined by Authors' and Somogyi Methods, Showing Relation Between Units of Two Procedures

Authors' method	Somogyi method	$\frac{\text{Somogyi unit}}{\text{Authors' unit}}$
units per 100 cc.	*units per 100 cc.*	
82.3	91.3	1.11
74.8	78.9	1.05
63.1	69.7	1.10
90.0	105.9	1.18
136.6	138.9	1.02
61.2	66.1	1.08
57.8	68.2	1.18
25.2	32.0	1.26
45.0	47.1	1.05
68.0	76.3	1.12
49.4	52.2	1.05
62.4	71.4	1.14
96.9	106.6	1.10
90.0	90.8	1.01
64.9	70.1	1.08
Average...............		1.10

(Table II) showed practically no variation over the range of concentration of substrate tested.

A second experiment was performed with use of a constant amount of starch with serial dilutions of enzyme solution. Both serum and urine were used as a source of enzyme. The results, as shown in Table III, indicate a satisfactory proportionality between enzyme concentration and the values obtained.

In a third experiment the relation of time of hydrolysis to amylase activity was studied. Enzyme and starch concentrations were held constant and the time of incubation was varied. Fig. 4 shows the results of this experiment. The curve obtained shows a straight line relationship during the 1st hour of hydrolysis, with some loss of activity between 1 and 2.5 hours of incubation. These results demonstrated the validity of the use of the 30 minute incubation period.

To show the relation of the authors' amylase unit to the unit of the Somogyi method, which is in wide-spread use, amylase determinations by both methods were made on the sera from fifteen human subjects. The results are recorded in Table IV. The amylase values by the Somogyi method are about 10 per cent higher than those obtained by our procedure. For practical purposes values by the two methods may be considered directly comparable.

SUMMARY

An amyloclastic method for the determination of α-amylase in blood and urine has been developed. The method makes use of the difference in the intensity of the color produced with iodine by a measured amount of soluble starch before and after hydrolysis by the enzyme. The method is rapid and has a high degree of accuracy.

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THE ANTIPHENYLALANINE EFFECT OF β-2-THIENYLALANINE FOR THE RAT*

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(Received for publication, December 27, 1948)

It has been demonstrated that β-2-thienyl-DL-alanine inhibits the growth of several different microorganisms and that the inhibition is reversed by the addition of phenylalanine to the medium (1–4). Recently this work has been extended to a study of the D and L isomers of thienylalanine, and it has been shown that in the case of three microorganisms the D isomer is without effect on growth, whereas the L isomer possesses twice the inhibitory activity of an equal weight of thienyl-DL-alanine (4).

Early experiments with rats (1) showed that thienyl-DL-alanine was unable to replace phenylalanine in the diet of growing animals. An attempt was then made to determine whether thienylalanine exerted an "antiphenylalanine" effect. These preliminary experiments (1) gave inconclusive results, although some indications of an antagonism were obtained, and a further investigation of this point therefore seemed necessary. The present communication is concerned with such an investigation of the effect of thienyl-DL-alanine, thienyl-D-alanine, and thienyl-L-alanine on the growth of rats.

EXPERIMENTAL

Young male albino rats purchased from the Rockland Farms were used for all the experiments. The basal diet, which contained a small amount of phenylalanine, had the following percentage composition: amino acid mixture (see Table I) 20.2, Osborne and Mendel salt mixture (5) 4, sucrose 55.8, hydrogenated vegetable oil 19, corn oil (Mazola) 1. The following amounts of vitamins were added to each kilo of diet: thiamine chloride 6 mg., calcium *dl*-pantothenate 40 mg., riboflavin 25 mg., nicotinic acid 25 mg., pyridoxine hydrochloride 6 mg., inositol 1 gm., *p*-aminobenzoic acid 200 mg., choline chloride 3 gm., vitamin A concentrate 40,000 units, vitamin D concentrate 4000 units, *dl*-α-tocopherol acetate 10 mg., and 2-methyl-1,4-naphthoquinone 1 mg. Control rats were maintained on this diet for periods up to 42 days and were found to exhibit slow growth.

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* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

The animals were maintained on the basal diet for periods of time varying from several days to several weeks. Thienyl-DL-alanine, thienyl-D-alanine, or thienyl-L-alanine was then added to the diet at the expense of an equal weight of sucrose, and the growth response was followed. After a suitable period DL-phenylalanine was added in addition to the thienylalanine. The growth rates and average daily food intakes are summarized in Tables II and III. In a few experiments the effect of L-tyrosine on the growth of animals fed a diet containing thienyl-DL-alanine was investigated.

TABLE I

Composition of Amino Acid Mixture

Amino acid	Per cent in diet	Amino acid	Per cent in diet
DL-Alanine	0.4	L-Lysine·HCl·H_2O	1.9
L-Arginine·HCl	0.6	DL-Methionine	0.7
L-Aspartic acid	0.2	DL-Phenylalanine	0.7
L-Cystine	0.2	L-Proline	0.2
L-Glutamic acid	2.0	DL-Serine	0.2
Glycine	2.8	DL-Threonine	1.4
L-Histidine·HCl·H_2O	0.7	L-Tryptophan	0.4
Hydroxy-L-proline	0.1	DL-Valine	2.0
DL-Isoleucine	1.8	$NaHCO_3$	1.3
DL-Leucine	2.6		
			20.2

RESULTS AND DISCUSSION

It was found in preliminary experiments that a diet containing 1 per cent thienyl-DL-alanine caused a loss in weight for a period of 4 to 10 days, which was followed by slow growth; this was also true for diets containing 1 per cent thienyl-D-alanine or 1 per cent thienyl-L-alanine. A higher level of the isomers was therefore tried.

In the experiments described in the present paper, a 2 per cent level was employed. The inclusion of any one of the three forms of thienylalanine in the diet at this level invariably resulted in an immediate and rapid decline in weight. Spontaneous resumption of growth on a diet containing 2 per cent thienylalanine was never encountered, even in cases in which feeding was continued for more than 4 weeks. The addition of phenylalanine to the diet containing 2 per cent thienylalanine counteracted the inhibition of growth. The extent of the inhibition produced by thienylalanine and of the reversal of this inhibition by phenylalanine was subject to individual variation, as is evident from the growth records given in Tables II and III.

TABLE II

Summary of Feeding Experiments with Thienyl-DL-alanine

Rat No.	Diet period	Supplement to basal diet	Food consumption	Weight change	Growth rate
	days		*gm. per day*	*gm.*	*gm. per day*
35	42	None	6.2	73–115	+1.0
38	35	"	5.6	83–114	+0.9
65	9	"	6.9	122–136	+1.6
	31	2% thienyl-DL-alanine	2.5	136– 66*	−2.3
63	10	None	6.2	112–122	+1.0
	40	2% thienyl-DL-alanine	2.3	122– 78	−1.1
49	10	None	8.9	103–128	+2.5
	8	2% thienyl-DL-alanine	2.4	128–105	−2.9
	12	2% " + 1.3% DL-phenylalanine	5.0	105–111	+0.5
	16	2% thienyl-DL-alanine + 1.8% DL-phenylalanine	7.0	111–135	+1.5
39	5	None	8.2	106–123	+3.4
	9	2% thienyl-DL-alanine	2.6	123– 97	−2.9
	9	2% " + 1.3% DL-phenylalanine	5.4	97–115	+2.0
	7	2% thienyl-DL-alanine	4.0	115– 99	−2.3
56	11	None	5.9	108–122	+1.3
	7	2% thienyl-DL-alanine	1.7	122– 99	−3.3
	20	2% " + 1.8% DL-phenylalanine	3.9	99– 99	0.0
41	5	None	7.8	118–135	+3.4
	21	2% thienyl-DL-alanine	3.7	135–108	−1.3
	4	2% " + 1.3% DL-phenylalanine	8.3	108–124	+4.0
36	11	None	6.6	72– 89	+1.5
	18	1% thienyl-DL-alanine	5.4	89–102	+0.7
	15	2% "	4.5	102– 92	−0.7
	5	2% " + 1.3% DL-phenylalanine	6.8	92–108	+3.2
48	13	None	5.5	108–126	+1.4
	11	2% thienyl-DL-alanine	2.2	126– 91	−3.2
	5	2% " + 1.3% DL-phenylalanine	3.2	91– 94	+0.6
	11	2% thienyl-DL-alanine + 1.8% DL-phenylalanine	3.5	94– 96	+0.2
45	19	None	6.0	118–139	+1.1
	8	2% thienyl-DL-alanine	1.3	139–108	−3.9
	10	2% " + 1.3% DL-phenylalanine	3.6	108–104	−0.4
	7	2% thienyl-DL-alanine + 1.8% DL-phenylalanine	1.7	104–103	−0.1

* Rat died.

TABLE III

*Summary of Feeding Experiments with Thienyl-*D*-alanine and Thienyl-*L*-alanine*

Rat No.	Diet period	Supplement to basal diet	Food consumption	Weight change	Growth rate
	days		*gm. per day*	*gm.*	*gm per day*
54	9	None	6.8	108-123	+1.7
	10	2% thienyl-D-alanine	2.7	123-101	-2.2
	11	2% " + 1.8% DL-phenylalanine	6.5	101-130	+2.6
61	11	None	6.2	118-132	+1.3
	7	2% thienyl-D-alanine	2.3	132-107	-3.6
	14	2% " + 1.8% DL-phenylalanine	6.0	107-130	+1.6
43	15	None	6.5	85-108	+1.5
	6	1% thienyl-D-alanine	4.8	108- 99	-1.5
	7	2% "	3.0	99- 89	-1.4
	9	2% " + 1.3% DL-phenylalanine	7.1	89-110	+2.3
47	7	None	5.7	121-135	+2.0
	5	2% thienyl-D-alanine	1.2	135-110	-5.0
	9	2% " + 1.8% DL-phenylalanine	4.0	110-104	-0.7
55	10	None	5.8	110-127	+1.7
	5	2% thienyl-L-alanine	1.4	127-108	-3.8
	16	2% " + 1.8% DL-phenylalanine	4.6	108-123	+0.9
40	15	None	8.7	86-116	+2.0
	6	1% thienyl-L-alanine	4.5	116-110	-1.0
	9	2% "	2.8	110- 98	-1.3
	10	2% " + 1.3% DL-phenylalanine	5.3	98-112	+1.4
59	9	None	5.9	116-124	+0.9
	7	2% thienyl-L-alanine	1.9	124-102	-3.1
	22	2% " + 1.8% DL-phenylalanine	4.3	102-102	0.0
35	42	None	6.2	73-115	+1.0
	4	1% thienyl-DL-alanine	6.0	115-115	0.0
	6	1% thienyl-L-alanine	5.5	115-113	-0.3
	9	2% "	4.0	113-103	-1.1
	7	2% " + 1.3% DL-phenylalanine	6.4	103-120	+2.4

It is interesting to note in connection with the inhibitory activity of thienyl-D-alanine for the rat that a diet containing D-phenylalanine in place of L-phenylalanine can support the growth of the rat (6). On the other hand, *Lactobacillus delbrueckii*, for which thienyl-D-alanine has been reported to be inactive (4), cannot utilize D-phenylalanine for growth (7). The other microorganisms for which thienyl-D-alanine has been shown to be inactive, *Saccharomyces cerevisiae* and *Escherichia coli* (4), do not require phenylalanine in the medium for growth.

An investigation was made of the effect of tyrosine on thienylalanine inhibition. In the case of four animals which had received the diet containing 2 per cent thienyl-DL-alanine for 4 to 6 days, the addition of 4 per cent L-tyrosine to the diet failed to cause any resumption of growth, although feeding of tyrosine plus thienylalanine was continued for 3 weeks. Feeding of 4 per cent L-tyrosine over the whole period of thienylalanine feeding failed to prevent the usual weight loss. The addition of 4 per cent L-tyrosine to the basal diet containing no thienylalanine did not decrease the rate of growth of control animals.

Although these results do not eliminate the possibility that tyrosine might be able to counteract the toxicity of thienylalanine under other dietary conditions, they do make it appear unlikely that such would be the case. For comparison, it may be noted that tyrosine is unable to reverse the toxicity of thienylalanine for *Saccharomyces cerevisiae* but can reverse the toxicity for *Escherichia coli* (3). In the case of the latter organism, evidence has been presented to show that thienylalanine prevents the synthesis of tyrosine from phenylalanine (8).

SUMMARY

The effect of β-2-thienyl-DL-alanine and the D and L isomers on the growth of rats fed a diet low in phenylalanine has been investigated. At a level of 2 per cent in the diet, all three forms of thienylalanine produced an inhibition of growth which could be counteracted by DL-phenylalanine. The antagonistic relationship between phenylalanine and thienylalanine, previously known only in microorganisms, has thus been extended to mammalian metabolism. The effect of tyrosine on the inhibition of growth produced by thienylalanine has also been investigated.

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THE CONVERSION OF S^{35}-HOMOLANTHIONINE TO S^{35}-CYSTINE IN THE RAT

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S-Bis(γ-amino-γ-carboxypropyl)sulfide, which we named homolanthionine, was found to be available to the rat for growth in lieu of cystine either on a low casein diet or on a diet in which the sole source of protein was supplied by a mixture of amino acids (1). This observation could not be easily explained on the basis of the assumption that homolanthionine was cleaved *in vivo* to yield homocysteine. Very poor growth was obtained with homolanthionine on diets which furnished choline and cystine, whereas with homocystine instead of homolanthionine good growth was obtained under similar conditions. Yet, homolanthionine stimulated the growth on diets containing minimal amounts of methionine. Since our preparation of homolanthionine consisted of a mixture of three isomers, it appeared possible that one of the isomers of homolanthionine yielded homocysteine *in vivo*, which in the presence of choline was converted to methionine, and thereby increased the amount of methionine available to the rat ingesting a diet containing minimal amounts of methionine. Such a formation of homocysteine from one of the isomers of homolanthionine would have to be small, indeed, since in the presence of choline and cystine, but with no methionine in the diet, this amount of formed methionine was insufficient to stimulate the growth, although maintenance of weight was secured.

These considerations of the data led us to the conclusion that at least one of the isomers of homolanthionine gave rise to cystine in the rat without the preliminary cleavage of the thioether to homocysteine. A working hypothesis was proposed which would tentatively explain such a transformation of homolanthionine to cystine (1).

Before proceeding with the elaboration of evidence in support of the proposed intermediates in such a transformation, it appeared necessary first to furnish proof that cystine is elaborated from homolanthionine. Since our hypothesis suggests that the sulfur of homolanthionine, and not necessarily its carbon chain, is available for the synthesis of cystine in the rat, the use of radioactive sulfur as a metabolic label appeared eminently suitable for testing directly this particular aspect of the hypothesis.

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The present report furnishes a direct proof that the sulfur of homolanthionine is available for the synthesis of cystine in the rat.

EXPERIMENTAL

A sample of S^{35}-S-benzylhomocysteine with the activity of 4 × 10^5 counts per minute per mg. was obtained through the courtesy of Dr. H. Tarver of the University of California. S^{35}-Homolanthionine was synthesized from this preparation by the previously described method (2). The compound was analytically pure, and it was free of either the thiol or the disulfide groups as far as could be determined by the existing methods. For feeding experiments the radioactive homolanthionine was diluted 20-fold with ordinary homolanthionine.

Three male rats (litter mates) of the Wistar strain, born and raised in the laboratory, were used. The animals were not previously experimented with. They were kept in individual metabolism cages, and water and food were allowed *ad libitum.* All rats were fed for 3 days the amino acid mixture diet (1), which was supplemented with 0.5 per cent choline chloride and 0.25 per cent of DL-methionine. On the 4th day the rats weighed 87 to 91 gm. Rat 272 then received, mixed with the diet, 0.8 gm. of S^{35}-homolanthionine per 100 gm. of food, Rat 273 received the same amount of ordinary homolanthionine, and Rat 274 was continued as a control on the same diet as was fed before. The feeding was continued for an additional 12 days, at the end of which time the experiments were terminated and the weight of all the rats and the weight of the food consumed were recorded.

The rats which ingested the homolanthionines were killed by a blow, and the hair of each animal was removed separately by an electric clipper. About 1 gm. of hair from each rat was collected. The hair was washed with soapy water, then with ethanol, followed by ethyl ether. The dry material from each rat was hydrolyzed separately with 30 times its weight of 1:1 mixture of formic and hydrochloric acids by refluxing for 17 hours. The acids were removed by distillation at reduced pressure, and the residue was dissolved in 10 ml. of 1 N HCl. From this point on a modification of the Vickery-White procedure (3) for the quantitative precipitation of cysteine as the cuprous mercaptide was used. After buffering the acid solution of the digest with acetate buffer to pH 4.5, the volume was adjusted to about 30 ml. with water and the solution was heated on a water bath to about 80°. Cuprous oxide was then added in small portions to the solution with mechanical agitation, until a small excess of the oxide was present. Reduction of cystine to cysteine prior to the addition of cuprous oxide was found to be unnecessary (4).

The precipitated material was allowed to settle for about an hour, removed by centrifugation, and washed three times with ethanol. The washed material was suspended in 30 ml. of 1 N HCl, and, while kept hot, was decomposed with a stream of H_2S. Copper sulfide was removed by centrifugation, washed with 1 N HCl, and the washings were added to the supernatant fluid. The volume of the solution was reduced to about 8 ml., and the cysteine was oxidized to cystine by careful addition of an alcoholic solution of iodine, avoiding an excess. The pH of the solution was then adjusted to 4.5 with dilute ammonia, and, after cooling in the refrigerator overnight, cystine was removed by centrifugation and recrystallized twice from dilute hydrochloric acid with dilute ammonia. For analysis, the isolated cystine was washed with ethanol, then with ether, and dried *in vacuo* at 100° over P_2O_5. About 74 mg. of pure cystine per gm. of rat hair were obtained. The product, on analysis, contained 26.59 per cent of sulfur; calculated for cystine, 26.67 per cent.

TABLE I

Data on Feeding Experiments, and Activities of Administered Homolanthionine and of Isolated Cystine

Rat No. (males)	Gain in weight in 12 days	Food consumed per day	Homolanthionine fed in 12 days		Activity of $BaSO_4$*	
			S^{35}	S^{32}	Homolanthionine	Cystine
	gm.	*gm.*	*mg.*	*mg.*	*counts per min.*	*counts per min.*
272	12	5.0	480		8900	130
273	13	5.1		490	0	0
274	3	3.0		None		

* Corrected for background which was 17 5 counts per minute.

The data on growth and the activities of the homolanthionine fed and of the isolated cystine are summarized in Table I. For radioactivity measurements the homolanthionine and the cystine were oxidized with concentrated nitric acid, followed by digestion with Denis' reagent (5), and the sulfates were isolated as $BaSO_4$. The measurements of the activities were made on $BaSO_4$ spread in an "infinitely thick" layer over an area of 5 sq. cm. under a mica window counter (6). We wish to express our thanks to Dr. S. Weinhouse of Temple University for extending the facilities and assistance in carrying out these measurements.

DISCUSSION

The data in Table I show that the activity of the isolated cystine was about 1.5 per cent of the total activity of the administered S^{35}-homolanthionine (130 of 8900 counts per minute). Considering the fact that the hair of the rat which was fed the radioactive homolanthionine was not removed before the administration of the compound was instituted, considerable dilution of S^{35}-cystine with preformed cystine must have taken place during a relatively short experimental period. In spite of this, the activity of the isolated cystine was sufficiently high to establish beyond reasonable doubt that the sulfur of homolanthionine is available to the rat for the synthesis of cystine.

In order to check the unlikely possibility that the radioactive homolanthionine found its way into the rat hair and was carried down as an impurity with inactive cystine during the isolation procedure, we added active homolanthionine to inactive cystine, dissolved the mixture in 1 N HCl, and carried out the isolation of cystine, as described before. The isolated cystine was inactive. There is no doubt, we believe, that the activity of the isolated cystine was not a result of contamination by radioactive homolanthionine within the hair of the rat. However unlikely, the possibility still remains that, in addition to radioactive cystine, radioactive homolanthionine or its metabolic products may have been present in the rat hair.

Of course, our data do not disclose any information regarding the fate of the carbon residue of homolanthionine or the origin of the carbon chain of cystine; nor do they elucidate the nature of the intermediates which are involved in the transformation of homolanthionine to cystine. Further work with isotopic labels in various positions of the homolanthionine molecule is clearly desirable. As we have pointed out previously (1), pure isomers of homolanthionine must be made available before one can definitely state which isomer is involved in the transformation of homolanthionine to cystine and, perhaps, to methionine.

That an artifact such as homolanthionine should give rise to cystine *in vivo* encourages further work on the problems of the proposed intermediates in this transformation (1), of the possible origin of homolanthionine from methionine, and, perhaps, its occurrence in natural products.

SUMMARY

S^{35}-Homolanthionine was synthesized and fed with food to rats. After 12 days, cystine was isolated from the hair. The isolated cystine was radioactive. The conclusion is drawn that the sulfur of homolanthionine is available to the rat for the synthesis of cystine.

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THE DETERMINATION AND THE URINARY EXCRETION OF CAFFEINE IN ANIMALS*

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Caffeine, its physiological actions, and its metabolic fate have been of great interest to the medical profession for many years because of the therapeutic value of the drug as a respiratory and circulatory stimulant, and its wide-spread consumption in beverages. Despite this, a suitable method for the detection and quantitative determination of small amounts of caffeine in body fluids has not been available. It is the purpose of this paper to present such a technique, along with certain observations concerning the excretion of caffeine by the dog and horse.

Chronologically, caffeine determination has passed from the stage of ultimate analysis of extractive residues early in this century (1, 2), through colorimetric procedures in the 1930's (3), to the use of ultraviolet spectrophotometry (4). The former are not applicable to urine and blood because of the impracticability of obtaining caffeine-containing extracts of sufficient purity for routine carbon and nitrogen analyses to yield significant results. The colorimetric procedure, with use of the murexide reaction, as described by Tanaka and Ohkubo (3), has, in our experience, failed to yield sufficient precision and specificity to allow its use in analysis of caffeine-containing urines. Ishler, Finucane, and Borker (4) have reported the use of the ultraviolet absorption spectrum of caffeine for its determination in coffees and crude caffeine preparations. This laboratory has been studying the ultraviolet absorption spectra of various methylated xanthines during the past year and has developed a technique based on these phenomena which allows detection of caffeine in amounts as small as 2.5 γ per cc. of urine. Ishler *et al.* have described the ultraviolet absorption spectrum of caffeine and found that it conforms to the Beer-Lambert law. We also confirm these findings and have obtained a molecular extinction of 10,800 ($\log I_0/I = 0.635$ at 273 mμ for 12.5 γ per cc.). Fig. 1 shows the spectrum of pure caffeine.

Analytical Method

Apparatus—A Beckman DU quartz photoelectric spectrophotometer, equipped with 10 mm. quartz cells, is used.

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Reagents—

1. Saturated aqueous solution of lead acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$).
2. Anhydrous powdered Na_2CO_3, reagent grade.
3. Powdered $NaHCO_3$, reagent grade.
4. Saturated aqueous $NaHCO_3$ solution.
5. 0.05 N HCl; prepared from redistilled HCl.
6. Specially purified $CHCl_3$. Reagent grade $CHCl_3$, washed serially with 10 per cent NaOH, concentrated H_2SO_4, two portions of distilled water, and then redistilled from glass.

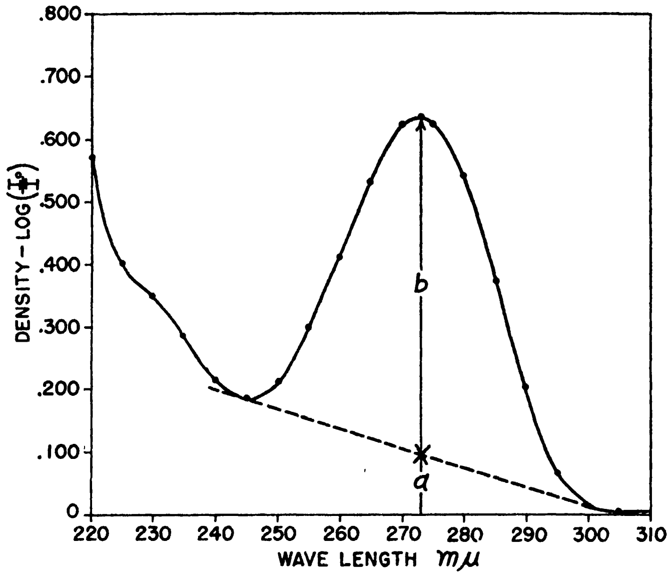

FIG. 1. Ultraviolet absorption spectrum of caffeine, concentration 12.5 γ per cc., in HCl at pH 3.

Procedure

Urine—A 40 cc. specimen of the urine is treated by the dropwise addition from a burette of a saturated solution of lead acetate until precipitation is complete. The solution is filtered and 1.0 gm. of $NaHCO_3$ is added to the filtrate. Na_2CO_3 is then added to pH 9.0 ± 0.5 (universal indicator paper). This results in the precipitation of excess lead, and the solution is again filtered. A 20 cc. aliquot of the filtrate is shaken serially with two 20 cc. portions of $CHCl_3$ in a separatory funnel. The combined $CHCl_3$ extracts are washed once with 5 cc. of 0.05 N HCl and dried by filtering through anhydrous sodium sulfate suspended on a cotton pledget in a small funnel. The solvent is then removed by vacuum distillation at room temperature. The final residue, usually crystalline if caffeine is present, is taken up in 10 cc. of warm water, filtered through cotton into a quartz cell, and the optical density recorded throughout the range 220 to 310 mμ. There will frequently be a small amount of water-insoluble amorphous material in the final residue (after chloroform evaporation), but it may safely be discarded, as we have found that this fraction, when dissolved in alcohol or chloroform, does not exhibit characteristic absorption bands in the ultraviolet.

Blood—A Folin-Wu filtrate is prepared with 15 cc. of blood, 75 cc. of water, 30 cc. of 10 per cent $Na_2WO_4 \cdot 2H_2O$, weight by volume, and 30 cc. of $\frac{2}{3}$ N H_2SO_4. 75 cc. of this filtrate are extracted directly with two 25 cc. portions of $CHCl_3$ and the extracts washed, dried, and further treated as in the procedure for urine, except that solution of the final residue for spectrophotometry is effected in 4 cc. of water.

Calculations

Urine—The optical density of the final washed urine extract is due to two components, *viz.* caffeine and other chromogens. To correct for the non-caffeine chromogens it is necessary to establish a "blank." This may be done experimentally by treating non-caffeine-containing urines by the analytical procedure. The magnitude of the optical density of such extracts of negative urine will vary considerably (Table I), depending principally on the relative amounts of chromogenic substances in the initial specimen.

The calculation is

$$C = 0.0246\ (d_X - d_B)\ (40 + L) \qquad (1)$$

where C = the concentration of caffeine in mg. per 100 cc., d_X = the optical density of the unknown (at 273 mμ); d_B = the optical density of the extract of the non-caffeine-containing specimen (at 273 mμ); L = the volume of lead acetate solution added in the first step of the extraction procedure.

With urine specimens containing more than about 5 γ per cc. of caffeine, it will be necessary to dilute the final extract before spectrophotometry. In this case d_X will be obtained by multiplying the observed density of the diluted extract by the dilution factor.

It has been found possible to apply an alternate procedure for estimating the blank in routine analyses of urine specimens in which an initial negative urine cannot be obtained. For this purpose it may be assumed that the absorption due to chromogens other than caffeine decreases as a straight line function in the range 245 to 300 mμ. The construction of such a blank absorption curve on an observed final extract spectrum is shown in Fig. 2 (line *AB*). The selection of the 300 mμ point (*B*) is based on the fact that caffeine has essentially zero absorption at 300 mμ (Fig. 1). The other point (*A*) is taken as the minimum of the observed curve in the region of 240 to 250 mμ. For pure caffeine (Fig. 1) the construction of such a line and observations of its ordinate at 273 mμ yield a value (*a*) which equals 15.7 per cent of the maximum absorption (*a* + *b*). In the unknown specimens the blank curve is constructed and its ordinate at 273 mμ is deducted from the observed maximum. The resulting value is then adjusted for the ratio of *b* to (*a* + *b*) (multiply by 1.19) and this value (d_y) substituted in Equation 1 for the ($d_X - d_B$) term; *i.e.*

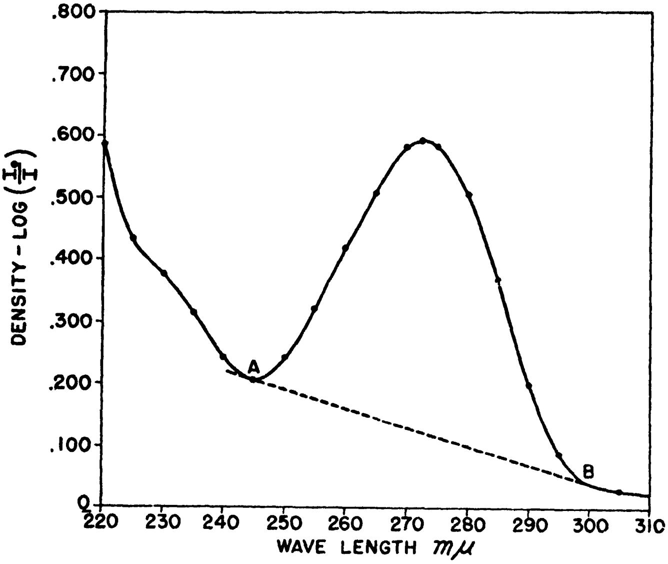

FIG. 2. Absorption spectrum of an extract of caffeine-containing urine, showing the construction of the blank absorption curve, *AB*.

$$C = 0.0246d_y\ (40 + L) \tag{2}$$

Blood—The same general considerations apply to blood as to urine with regard to alternate methods of estimating the blank (absorption due to chromogens other than caffeine). The equations are

$$C = 1.05(d_X - d_B) \tag{3}$$

$$C = 1.05d_y \tag{4}$$

Comments

A series of ultraviolet absorption spectra typical of those obtained in analysis of urine is shown in Fig. 3, where the spectra of the final extracts of the 1, 2, and 8 hour specimens from Experiment II (Table III) are plotted. The volume of urine sample and the final volume of extract submitted to spectrophotometry were 25.0 cc. each, dilution of the extract to this final volume being necessary because of the concentration of caffeine present. The blank curve (0 hour) was obtained in this case from urine collected immediately before the caffeine was administered.

Table I illustrates the significance, in the final analytical results, of the two alternate methods of estimating the non-caffeine chromogens in the extract subjected to spectrophotometry. These determinations are selected from the experimental results reported subsequently in this paper. They exemplify urine and blood specimens in which the caffeine concentrations varied within wide limits. Examination of the results shows that there is close agreement between the two methods when dog urine is analyzed. (The limits were from 96 to 104 per cent and the range of caffeine concentration was from 0.24 to 4.7 mg. per 100 cc. of urine.) This is to be expected when the magnitude of the blank is low, as is generally characteristic of dog urine. In the case of horse urine, the disparity is greater, with one pair of results differing by 38 per cent. This, however, was in a specimen with very low caffeine concentration and a relatively high non-caffeine-chromogen content (0.25 mg. per 100 cc. of caffeine and d_B = 0.232).

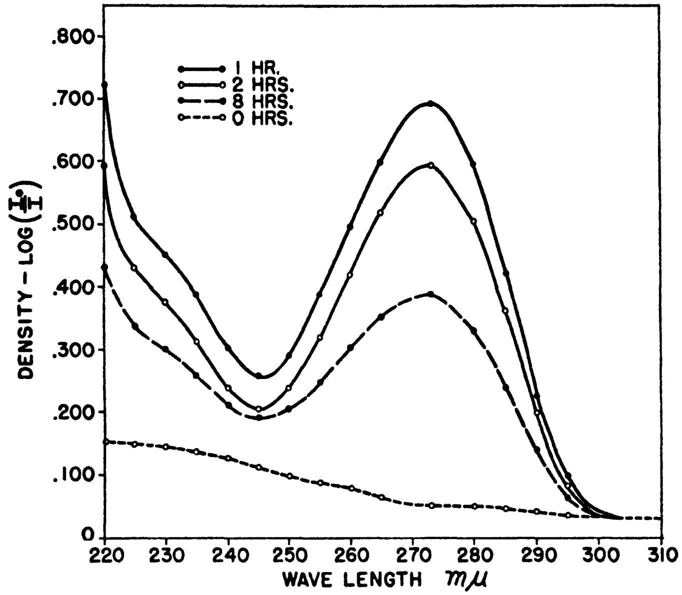

FIG. 3. Absorption spectra of extracts of urine from caffeine-stimulated dog. 25 cc. samples; final extracts diluted to 25 cc.

Both theobromine and theophylline have absorption spectra which are quite similar to, though not identical with, that of caffeine and theoretically would interfere with the determination of caffeine. In actual extractions, approximately 35 per cent of theobromine present in original solutions (concentrations of 0.25 and 5.0 mg. per cent) was recovered in the final residue. With theophylline in similar concentrations, less than 2.5 per cent was recovered, and it is judged that it would not interfere with caffeine determination except under very extraordinary conditions.

TABLE I

Effect of Calculating Results with Estimated Blank

d_X	d_B	Concentration calculated with d_B	Concentration calculated by estimated blank	Divergence*	Remarks
		mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	
0.414	0.232	0.21	0.13	−38	(See Table II) Horse urine, 0.25 mg. caffeine added per 100 cc.
4.070	0.232	4.3	4.3	0	Horse urine, 5.0 mg. caffeine added per 100 cc.
0.313	0.093	0.24	0.23	−4	Dog urine, 0.25 mg. caffeine added per 100 cc.
4.340	0.093	4.7	4.9	+4	Dog urine, 5.0 mg. caffeine added per 100 cc.
0.830	0.170	0.73	0.64	−12	(See Table IV, Experiment III) Horse urine. 1st hr.
0.756	0.170	0.65	0.65	0	2nd hr.
0.964	0.170	0.88	0.71	−19	4th "
1.115	0.330†	0.87	0.69	−21	(See Table IV, Experiment IV) Horse urine. 1st hr.
1.549	0.342†	1.3	1.4	+8	10th hr.
1.424	0.352†	1.2	1.1	−8	19th "
1.239	0.094	1.3	1.3	0	(See Table III, Experiment II) Dog urine. 1st hr.
0.694	0.094	0.66	0.65	−2	8th hr.
0.409	0.133	0.29	0.26	−10	(Experiment III) Horse blood. 10 min. after administration
0.400	0.133	0.28	0.28	0	70 " " "
0.660	0.114	0.57	0.57	0	(Experiment I) Dog blood. 1st hr. after administration
0.598	0.114	0.50	0.46	−8	2nd hr. after administration

* Ratio of the difference between the two results and the result based on calculations with d_B.

† Same "blank" urine specimen but analyzed on successive days. Note increase in d_B with increasing age.

The acid washing of the combined chloroform extracts serves to remove certain organic bases from the solution and thereby prevent their interference in later spectrophotometry of the caffeine. (Among these are strychnine, benzedrine, and nicotine.) Further studies are in progress with the intention of determining these substances, should they be present in the specimen of urine examined. If the acid extract is to be examined spectrophotometrically, a preliminary wash of the chloroform is advisable (4 cc. of saturated sodium bicarbonate solution).

Results

In Vitro—Varying amounts of caffeine were added to samples of horse and dog urine and the specimens analyzed by the above technique. The recoveries are shown in Table II.

TABLE II

Recovery of Caffeine Added to Urine

Horse			Dog		
Known concentration	Found concentration	Recovery	Known concentration	Found concentration	Recovery
mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
0.25	0.21	84	0.25	0.24	96
5.0	4.3	86	1.4	1.3	93
			5.0	4.8	96

Dogs—Female greyhounds were prepared for catheterization by surgical exposure of the urethral orifice and the incisions allowed to heal. Caffeine was administered intravenously and by stomach tube and urine specimens collected at intervals and analyzed for caffeine. The results are presented in Table III.

Blood specimens taken at 1 and 2 hours after the intravenous dosage were analyzed and showed 0.57 and 0.50 mg. per 100 cc. of caffeine respectively.

It is apparent at once that only a very small fraction of an administered dose of caffeine is recoverable in the urine. Further, the magnitude of this is influenced by the relative diuresis. Thus in Experiment II, water diuresis occurred because of the administration of 625 cc. of water with the caffeine, and 7.5 mg., or 3.0 per cent, of the administered dose were recovered in 6 hours. In Experiment I, in which water was not given by stomach tube, only 0.7 per cent was recovered in 6 hours.

Horse—The elimination of caffeine by a mare was studied after intravenous and oral administration of the drug. The urine specimens were collected through a large Foley catheter which was kept clamped between collections of the various samples. Table IV shows the results of these studies.

TABLE III

Concentration and Recovery of Caffeine in Dog Urine

Dosage of caffeine	Time after administration	Urine volume	Caffeine concentration	Total caffeine	Remarks
mg. per kg.	*hrs.*	*cc.*	*mg. per 100 cc.*	*mg.*	
9.75	1	28	1.4	0.39	Experiment I. Weight 20.5 kilos; caffeine sodium benzoate, 400 mg. intravenously
	2	14	1.3	0.18	
	4	27	2.0	0.54	
	6	24	1.2	0.29	
Total...		93		1.40	0.7% recovery
10.0	1	96	1.3	1.2	Experiment II. Weight 25.0 kilos; caffeine citrate, 0.5 gm. in 625 cc. water *per os;* water *ad libitum*
	2	294	1.1	3.2	
	4	224	1.0	2.2	
	6	114	0.82	0.9	
	8	150	0.66	1.0	
	12	250	0.88	2.2	
	24	537	0.37	2.0	
Total...		1665		12.7	5.1% recovery

TABLE IV

Concentration and Recovery of Caffeine in Horse Urine

Time after administration	Urine volume	Caffeine concentration	Total caffeine recovered	Remarks
hrs.	*cc.*	*mg. per 100 cc.*	*mg.*	
1	355	0.73	2.6	Experiment III. 3.0 gm. caffeine sodium benzoate intravenously; mare weighing about 430 kilos
2	200	0.65	1.3	
3	410	0.65	2.7	
4	160	0.88	1.4	
Total.....	1125		8.0	0.5% recovery
1	330	0.87	2.9	Experiment IV. 3.0 gm. caffeine alkaloid orally in gelatin capsule; same mare as in Experiment III; recovery in first 4 hrs., 0.5%
2	550	1.1	6.1	
3	135	1.6	2.2	
4	250	1.5	3.8	
5	660	1.4	9.2	
6	38	1.5	0.6	
8	760	1.7	12.9	
10	540	1.3	7.0	
12	800	0.92	7.4	
15½	835	1.1	9.2	
19	1335	1.2	16.0	
21	370	1.1	4.1	
24	840	0.71	6.0	
Total.....	7443		87.4	2.9% recovery

Examination of these results shows that after intravenous administration of caffeine the urine level of the drug is approximately the same in each of the first 4 hourly specimens. Blood samples taken 10 and 70 minutes after injection of the drug were analyzed and showed 0.29 and 0.28 mg. per 100 cc. respectively. These are to be contrasted with the 1st hour urine which contained 0.73 mg. per 100 cc., essentially 3 times that in the blood. The same general relationship was found in the dog in Experiment I (see Table III).

After oral administration of caffeine to the horse, the concentration of caffeine in the urine rose progressively during the first 3 hours, remained relatively constant through the 8th hour, and then declined gradually to 0.71 mg. per 100 cc. at the end of 24 hours. At 48 hours, a urine specimen contained approximately 0.17 mg. per 100 cc. of caffeine. Thus, with dosage of the magnitude described herein, it is readily possible to detect caffeine in any single urine specimen within the first 24 hours after administration. With the horse, as with the dog, only a small fraction of a given dose of caffeine was recovered in 24 hours.

SUMMARY

1. A method for rapid detection and determination of caffeine in urine and blood, with an ultraviolet spectrophotometric technique, is described. It is applicable to urinary concentrations of the drug as low as 2.5 γ per cc.

2. The procedure allows the isolation and detection of caffeine in the presence of other commonly used stimulants and the strongly basic alkaloids.

3. Caffeine appears promptly in the urine of dogs and horses after the oral or intravenous administration of the drug. It continues to be excreted in the urine for at least 24 hours after administration.

4. The urinary recovery of orally administered caffeine in the first 24 hour period after dosage was 2.9 per cent in a horse and 5.1 per cent in a dog.

We are indebted to Dr. J. A. McComb, of the Division of Biologic Laboratories, Massachusetts Department of Public Health, for cooperation in the horse experiments reported herein.

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THE INFLUENCE OF MAGNESIUM AND COBALT ON THE INHIBITION OF PHOSPHATASES OF BONE, INTESTINE, AND OSTEOGENIC SARCOMA BY AMINO ACIDS*

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In previous papers some of the characteristics and the extent of the inhibition of alkaline phosphatase activity by α-amino acids were evaluated (1). The first concern of the present study was to investigate the influence of magnesium ion on this inhibition. Since the results which were obtained in this connection indicated the possibility that α-amino acids might exert their inhibitory action through combination with an essential metal component of the enzyme, the studies were extended to several other phases: (*a*) the effect of magnesium on the inhibition of bone and intestinal phosphatases by cyanide ion; (*b*) the influence of cobalt alone and in combination with magnesium upon the inhibition of these phosphatases by amino acids; (*c*) the effect of sodium azide on the activity of phosphatase. Finally, it was considered of value to determine the extent to which the phosphatase of human osteogenic sarcoma resembled that of rat bone with respect to the inhibition by amino acids and the influence of magnesium and cobalt on this inhibition.

EXPERIMENTAL

The methods for preparing phosphatase extracts and for purifying them by dialysis have been described previously in detail (2). In the present work, rat bone, rat intestinal, and human osteogenic sarcoma phosphatases were made by this method. The degree of purity was defined in units as the ratio of the reaction velocity, in micrograms of inorganic phosphorus liberated as phosphate per cc. of hydrolysis per minute, to the total nitrogen content, expressed in mg., of the enzyme contained in 1 cc. of the hydrolysis mixture. The purity of rat bone phosphatase preparations usually averaged about 20 units at 24.80°, whereas that of intestinal phosphatase preparations ranged from 40 to 100 units at this temperature. The purity of one of the osteogenic sarcoma phosphatases, preparation MOSB,

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was 344 units at a temperature of 32.20°. This osteogenic sarcoma phosphatase preparation was further purified by a method which was essentially that of Robison and his coworkers (3, 4) and which involved precipitation of the dialyzed preparation with an alcohol-ether mixture, extraction of the precipitate with dilute alcohol, and precipitation of this extract with alcohol containing 0.25 per cent sodium acetate. The purity of this preparation was 1240 units at 32.20°.[1]

In most of the experiments reported in the present paper, the concentration of enzyme was 12.5 per cent by volume of the hydrolysis mixture; that of the substrate, sodium β-glycerophosphate, was in the maximal range, 0.0254 M. The concentration of the buffer, sodium diethyl barbiturate, was 0.5 gm. per 100 cc. of hydrolysis mixture or 0.024 M. Small amounts of 0.1 N or 1.0 N sodium hydroxide or hydrochloric acid were added to yield the required pH range. Magnesium, cobalt, amino acid, or any other substance or combination of substances pertinent to the particular experiment were added to the substrate-buffer mixture and allowed to remain at room temperature for about 5 to 10 minutes and at the temperature of the bath for another 5 minutes before the addition of the enzyme which had also been brought to the temperature of the bath. It was found, however, that there was no significant difference in reaction rate if the substrate were added last to a mixture of the enzyme and the other constituents. In the experiments dealing with the effect of cobalt, or magnesium, of both metals conjointly, or of the amino acids, cyanide, etc., each determination of phosphatase activity was carried out, as previously described (2), at optimal alkaline pH which was assured by running a series of hydrolyses constituting a very closely spaced pH-activity curve in and about the optimal range.

The reaction velocities were determined from the amount of phosphorus liberated as inorganic phosphate at three time intervals spaced as equally apart as possible during the initial portion of the hydrolysis and were expressed as micrograms of inorganic phosphorus liberated per cc. of hydrolysis mixture per minute. The time during which these observations were made depended upon the activity of the preparation and varied from 6 to 90 minutes. As previously pointed out (1) this initial portion was practically always of zero order. However, in the reactions in the presence of cobalt and amino acid at very high pH levels on the alkaline side of the optimum, the activity of the enzyme decreased and no strictly zero order portion could be obtained; in these latter instances the three readings were averaged. The liberated inorganic phosphate was determined by the method of Fiske and Subbarow (7) after assurance had been gained that the colorimetric readings were not affected by any of these substances at the concentrations employed in the experiments. The hydrolyses were conducted in a water thermostat regulated to within ±0.03°, at 24.80° during the spring months and 32.20° during the summer months. In those experiments in which the relationship between pH and enzyme activity was of interest, the pH was determined electrometrically by means of a Beckman pH glass electrode meter standardized with buffers of pH 8.0, 9.0, and 10.0. The α-amino acids were of reagent grade and were from the following sources: Fisher, Eastman Kodak, and Merck. The α-amino acids were made up in solutions adjusted to a pH of about 9.0, or else the necessary amount of sodium hydroxide was added to take into account the buffering capacity of the amino acids. Magnesium was used as $MgCl_2 \cdot 6H_2O$ (Fisher) and the cobalt as $Co(NO_3)_2 \cdot 6H_2O$ (Baker's Analyzed reagent).

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[1] It was of interest to compare the purity of our preparations with that of Albers and Albers (5) who developed procedures for the purification of kidney phosphatase. Our values for the activities of the preparations used in the present work were recalculated in terms of Martland and Robison units (3, 4); in this calculation the energy of activation of bone phosphatase (6) and the relation between total N content and dry weight were taken into account. Martland and Robison (3) obtained crude preparations of bone phosphatase with an activity of 0.7 unit per mg. of dry weight and purified preparations with an activity of 5.7 units per mg. of dry weight. The purest kidney phosphatase preparations of Albers and Albers (5) had an activity of about 50 to 60 units per mg. of dry weight. In the present work, the activities of an intestinal phosphatase, RIQ-d, of a bone phosphatase, RBQ-d, and of an osteogenic sarcoma phosphatase, MOSB-d1, were, respectively, 8.6, 2.2, and 25 Martland and Robison units per mg. of dry weight. The activity of the purified osteogenic sarcoma phosphatase, MOSB-P, was 125 units per mg. of dry weight, or about 2 to 3 times that of the purest preparations of kidney phosphatase obtained by Albers and Albers (5).

Other techniques are described in connection with specific experiments.

Results

Influence of Magnesium on Inhibition of Bone Phosphatase by Amino Acids—Table I shows several typical series of experiments on the inhibition of the activity of rat bone phosphatase by amino acids in the absence of any added magnesium and in the presence of what would ordinarily be an optimal concentration of magnesium, 0.0125 M. It may be seen that the extent of activation by magnesium decreased as the concentration of inhibiting amino acid was increased. Indeed, at the higher concentrations of amino acids, magnesium exerted an additional inhibitory effect.

The magnitude of the activating effect of magnesium with increasing concentrations of amino acid also appeared to depend on the extent to which the bone phosphatase extract had been dialyzed. Thus rat bone phosphatase, preparation RBR-d2 (not shown in Table I), was dialyzed for 4 days instead of the usual 2 days and showed, accordingly, a much greater acceleration, 280 per cent, in the presence of 0.0125 M magnesium. At concentrations of 0.0625 M and 0.156 M glycine, the activation due to magnesium was reduced to 150 and 140 per cent, respectively. When a small amount of magnesium was added to the dialyzed preparation so as to yield a final concentration of 0.0000125 M and thus simulate a less completely dialyzed preparation, the activation by 0.0125 M magnesium was 52 per cent in the absence of any amino acid and 8 per cent at a concentration of 0.156 M glycine.

TABLE I

Activation of Bone Phosphatase by Magnesium at Varying Concentrations of Amino Acids

The conditions of the reaction are as described in the text; temperature, 24.80°.

Phosphatase preparation	Amino acid	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.		Activation
		No added magnesium	0.0125 M magnesium present	
		γ	γ	*per cent*
RBP-d	None	0.39	0.55	41
	0.00625 M glycine	0.41	0.56	36
	0.0245 " "	0.33	0.34	3
	0.0594 " "	0.28	0.23	−17
RBO-d	None	0.66	1.00	52
	0.0125 M hydroxyproline	0.70	0.91	44
	0.0375 " "	0.63	0.66	5
	0.125 " "	0.36	0.30	−17
RBR-d	None	0.49	0.64	31
	0.0375 M L-glutamic acid	0.52	0.57	10
	0.0813 " " "	0.46	0.47	2
	0.125 " " "	0.40	0.36	−10
	0.250 " " "	0.28	0.11	−61

The results which have just been presented would indicate that the usual relationship between phosphatase activity and magnesium concentration did not hold for bone phosphatase at inhibiting concentrations of amino acids. This fact is demonstrated more completely in Table II. In the absence of any added amino acid, the rat bone phosphatase preparation, RBQ-d, shows the well established increase of activity with increase of magnesium ion until an optimal concentration of about 0.01 M magnesium was reached; thereafter, at the higher concentration of 0.125 M magnesium, decrease of activity resulted (8, 9). However, at a concentration of 0.0117 M L-histidine, the highest phosphatase activity was found at the minimal concentration of magnesium, 0.00001 M (that due to the content of the dialyzed enzyme preparation), and the activity of the enzyme decreased as the concentration of magnesium was increased. Thus, at what would ordinarily be an optimal concentration of magnesium, 0.0125 M, and would yield a maximal activity, the reaction velocity was *decreased* to 42 per cent of the velocity in the absence of any added magnesium.

Influence of Magnesium on Inhibition of Intestinal Phosphatase by Amino Acids—Table III shows several typical series of experiments on the inhibition of rat intestinal phosphatase preparations by amino acids in the absence of any added magnesium and in the presence of 0.0125 M magnesium. In contrast to the effect on bone phosphatase, the extent of the activating action of magnesium remained constant, within experimental variation, as the concentration of amino acid was increased. That the usual relationship between magnesium concentration and phosphatase activity holds for intestinal phosphatase as well at high concentrations of amino acids as in the absence of any added amino acids is shown in Table IV.

TABLE II

Influence of Magnesium Concentration on Activity of Bone Phosphatase in Absence and Presence of L-Histidine

The conditions of the reaction are as described in the text; temperature, 24.80°. The bone phosphatase preparation, RBQ-d, was used.

Concentration of added magnesum	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.	
	No amino acid	0.0117 M L-histidine
M	γ	γ
0.0*	0.387	**0.108**
0.00125	0.462	0.093
0.0125	**0.524**	0.045
0.0375	0.513	0.034
0.125	0.438	0.028

The maximal reaction velocities are printed in bold-faced type.

* The concentration of magnesium due to the content of dialyzed phosphatase preparation was 0.00001 M.

Influence of Magnesium on Inhibition of Phosphatases by Cyanide—The formation of complexes between heavy or transitional metal ions and amino acids has been demonstrated by a number of investigators (10–16). Zörkendörfer (11) submitted evidence for the formation of a complex between magnesium and glycine and, possibly, other amino acids as well. The results which we have presented gave rise to two possible explanations; namely, either that the inhibition was due to the formation of an undissociated compound between amino acid and magnesium, or else that amino acids, by reacting with some metal component of the phosphatase other than magnesium, not only decreased the activity of the enzyme but, in the case of bone phosphatase, also affected in some manner the capacity of magnesium to activate the phosphatase. The first possibility was contradicted by the data of Tables II and IV, for, if inhibition were due to the removal of magnesium to form an undissociated magnesium-amino acid

TABLE III

Activation of Intestinal Phosphatase by Magnesium at Varying Concentrations of Amino Acids

The conditions of the reaction are as described in the text; temperature, 24.80°.

Phosphatase preparation	Amino acid	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.		Activation
		No added magnesium	0.0125 M magnesium present	
		γ	γ	*per cent*
RIR-d	None	0.25	0.85	240
	0.0125 M L-glutamic acid	0.17	0.69	300
	0.0375 " " "	0.11	0.45	310
	0.075 " " "	0.07	0.30	330
	0.100 " " "	0.065	0.23	250
	0.0031 M L-histidine	0.17	0.58	240
	0.0125 " "	0.063	0.26	310
RIP-d	None	0.20	0.36	79
	0.0375 M L-hydroxyproline	0.16	0.26	63
	0.125 " "	0.10	0.17	70

TABLE IV

Influence of Magnesium Concentration on Activity of Intestinal Phosphatase in Absence and Presence of L-Histidine

The conditions of the reaction are as described in the text; temperature, 24.80°. Rat intestinal phosphatase, RIP-d, was used.

Concentration of added magnesium	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.	
	No amino acid	0.0117 M L-histidine
M	γ	γ
0.00	0.197	0.082
0.00125	0.282	0.082
0.0125	**0.360**	**0.120**
0.0375	0.334	**0.122**
0.125	0.260	0.100

The maximal reaction velocities are printed in bold-faced type.

compound, then an increase in the concentration of magnesium should have counteracted the inhibitory effect of the amino acid. This, however, was not the case. The second possibility suggested experiments with cyanide ion, since cyanide is known to form complexes with heavy or transitional metal ions (17) and is also a well established inhibitor of several enzymes, particularly those known to contain a metal component (18–22).

Table V shows that 0.0125 M magnesium increased the activity of bone phosphatase when no cyanide was present but *decreased* it markedly at a concentration of 0.00125 M cyanide. This result went hand in hand with the finding (Table VI) that, in the presence of cyanide, the highest bone phosphatase activity was found at the minimal concentration of magnesium and that the activity of the enzyme decreased as the concentration of magnesium was increased to what, in the absence of cyanide or amino acid, would have been an optimal concentration. In contrast, the degree of the activating effect of magnesium on intestinal phosphatase was unaffected by the presence of cyanide. The concentration of magnesium which gave maximal activity of the intestinal phosphatase was the same, 0.0125 M, in the presence as well as in the absence of cyanide (Table VI).

TABLE V

Activation of Bone and Intestinal Phosphatase by Magnesium at Varying Concentrations of Cyanide

The conditions of the reaction are as described in the text; temperature, 24.80°.

Preparation	Concentration of cyanide	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.		Activation
		No added magnesium	0.0125 M magnesium	
	M	γ	γ	*per cent*
Rat bone phosphatase, RBQ-d	0.0	0.387	0.524	36
	0.000125	0.320	0.407	27
	0.00125	0.112	0.069	−38
Rat intestinal phosphatase, RIQ-d	0.0	0.35	0.82	135
	0.000125	0.28	0.65	130
	0.000375	0.20	0.42	110
	0.00125	0.059*	0.138	135

* It was difficult to obtain precise optimal velocities in this instance, since it was found that the rate increased during the course of hydrolysis at less alkaline pH levels. Incubation experiments showed that this was probably due to loss of HCN from the reaction mixtures and that this loss was naturally most marked in very slow reactions. The value given was based on the earliest point in the course of the hydrolysis.

Effect of Sodium Azide on Bone and Intestinal Phosphatase Activity—Keilin (21, 22) found that sodium azide, like cyanide, strongly inhibited tissue respiration and the activities of cytochrome oxidase, catechol oxidase, catalase, peroxidase, and uricase. For example, peroxidase was inhibited 70 per cent by 0.003 M azide and liver catalase was completely inhibited by 0.001 M azide. On the other hand, xanthine oxidase which was inhibited by cyanide was not affected by azide. Table VII shows that the inhibitory effect of sodium azide on bone and intestinal phosphatases at optimal pH and 0.0125 M magnesium was negligible.

TABLE VI

Influence of Magnesium Concentration on Activity of Bone and Intestinal Phosphatase in Absence and Presence of Cyanide

The conditions of the reaction are as described in the text; temperature, 24.80°. Rat bone phosphatase, RBQ-d, and rat intestinal phosphatase, RIQ-d, were used.

Concentration of magnesium	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.			
	Bone phosphatase, RBQ-d		Intestinal phosphatase, RIQ-d	
	No cyanide	0.00125 M cyanide	No cyanide	0.00125 M cyanide
M	γ	γ	γ	γ
0.00	0.387	**0.112**	0.353	0.059
0.00125	0.462	0.092	0.64	0.098
0.0125	**0.524**	0.069	**0.82**	**0.138**
0.125	0.438		0.72	0.080

The maximal reaction velocities are printed in bold-faced type.

TABLE VII

Effect of Sodium Azide on Bone and Intestinal Phosphatase Activity

The conditions of the reaction are as described in the text. The concentration of magnesium was 0.0125 M in all hydrolyses. Reactions were run at optimal pH and temperature of 32.20°. Rat bone phosphatase, RBR-d3, and rat intestinal phosphatase, RIR-d1, were used.

Concentration of sodium azide	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.		Inhibition of	
	Bone phosphatase	Intestinal phosphatase	Bone phosphatase	Intestinal phosphatase
M	γ	γ	*per cent*	*per cent*
0.0	1.03	0.92		
0.00125	0.97	0.95	6	−3
0.0125	0.96	0.88	7	4
0.125	0.77	0.67	25	27

Effect of Cobalt on Inhibition of Phosphatase Activity by Amino Acids—We have already indicated the possibility that amino acids, by reacting with a metal component of bone phosphatase other than magnesium, might in some as yet undefined manner decrease the capacity of magnesium to activate this enzyme. It was therefore relevant to determine whether the activating effect of magnesium on bone phosphatase which had been inhibited by amino acids could be restored by supplying this metal component or some presumably similar metal component to the bone phosphatase-substrate system.

There are no direct data concerning the nature or even the existence of the metal component of bone or intestinal phosphatase. Massart and Vandendriessche (23), using spectographic techniques, have reported the occurrence of zinc, copper, iron, manganese, and magnesium in a purified kidney phosphatase preparation made by the method of Albers and Albers (5). In view of the lack of direct information concerning the nature of the metal component of bone or intestinal phosphatase, it was decided to determine whether there was a metal ion which acted as an activator of these phosphatases and which could also act to restore the magnesium activation of bone phosphatase inhibited by amino acids. Massart and Vandendriessche (23), Bamann and Heumiller (24), Roche and van Thoai (25), and Cloetens (26) have shown that manganese and cobalt activate phosphatase. In the present investigations, it was found that either manganese alone or cobalt alone, in the absence of added magnesium or amino acids, caused definite activations of bone and intestinal phosphatase preparations made as described earlier in this paper. Preliminary series of experiments with a bone phosphatase preparation, RBR-d4, and an intestinal phosphatase preparation, RIS-d, at varying concentrations of cobalt showed that maximal activation occurred at 0.001 to 0.01 M cobalt. A concentration of 0.00125 M cobalt was used in subsequent experiments.

Before testing the capacity of cobalt to restore the activating effect of magnesium on bone phosphatase inhibited by amino acids, it was necessary to determine the effect of cobalt alone on phosphatase activity in the presence of various inhibiting concentrations of amino acids. The bearing upon our results of Burk's recent work (14–16) concerning the formation of cobalt-amino acid complexes and their reversible oxygenation and irreversible oxidation will be discussed later.

Table VIII shows two typical series of experiments in which, as the concentration of amino acid was increased, the extent of activation by cobalt was decreased. This decrease was slight in the case of bone phosphatase and marked for intestinal phosphatase. Other instances of the decrease in the activating effect of cobalt as the concentration of amino acid was increased will be presented later in conjunction with other data.

The extent of the activating effect of cobalt at varying concentrations of amino acids was determined at optimal pH, in accordance with the technique which was outlined earlier in this paper. It was observed that in the action of bone phosphatase in the presence of cobalt and inhibiting amino acid there appeared to be a shift from the usual optimal range of about 9.0 to 9.5 towards a more alkaline region. The extent of this shift was determined more precisely electrometrically. Fig. 1 shows that the optimal pH range for the activity of the bone phosphatase preparation, RBR-d4, at a concentration of 0.00125 M cobalt was 9.4 to 9.6. In the presence of this concentration of cobalt and 0.125 M glycine, the optimal pH was shifted to a value of 10.4 to 10.6. The shift when 0.0125 M L-histidine was used was less marked, namely, to about 9.9. No such shift of the pH optimum towards the alkaline side was observed when the activity of intestinal phosphatase was determined in the presence of cobalt and amino acids (Fig. 1, *B*).

TABLE VIII

Activation of Bone and Intestinal Phosphatase by Cobalt at Varying Concentrations of Amino Acids

The reaction conditions are as described in the text; temperature, 32.20°. Rat intestinal phosphatase, RIR-d1, and rat bone phosphatase, RBR-d1, were used.

Phosphatase preparation	Amino acid	Reaction velocity, as P liberated as inorganic phosphate per cc. per min		Activation due to cobalt
		No added cobalt	0.00125 M cobalt present	
		γ	γ	*per cent*
Bone	None	0.56	0.68	21
	0.00124 M L-histidine	0.43	0.62	44
	0.0031 " "	0.35	0.44	31
	0.0093 " "	0.20	0.25	25
	0.0124 " "	0.15	0.16	7
Intestinal	None	0.25	0.83	230
	0.0125 M L-glutamic acid	0.17	0.36	110
	0.0375 " " "	0.11	0.11	0

Influence of Conjoint Presence of Cobalt and Magnesium on Inhibition of Bone Phosphatase Activity by Amino Acids—It has already been amply demonstrated in the course of this work that at inhibiting concentrations of amino acids the extent of the activation of bone phosphatase by either magnesium or cobalt alone was decreased, particularly so with the former metal. Table IX shows that at these concentrations of amino acids the extent of activation of bone phosphatase by magnesium and cobalt *present conjointly* was much greater than that by either magnesium or cobalt alone and often as great as, or greater than, the extent of activation of bone phosphatase when no inhibiting amino acid was present. In other words, cobalt restored, either partially or completely, the activating effect of magnesium on bone phosphatase which was inhibited by amino acids, and tended to counteract this inhibition. For example, the inhibitions produced by 0.0625 M glycine in the absence of any added metal ion, in the presence of magnesium alone, and in the presence of cobalt alone were,

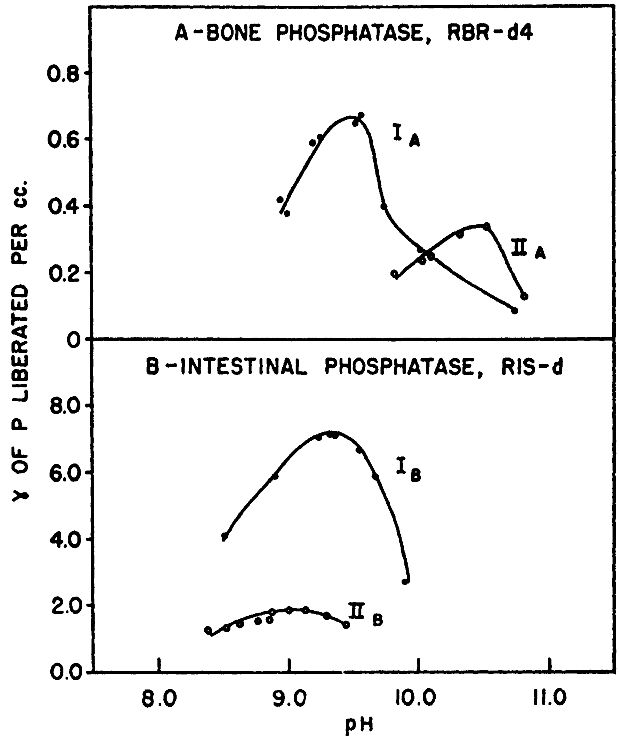

FIG. 1. The effect of glycine on the pH optimum of cobalt-activated bone and intestinal phosphatases. All reaction mixtures contained substrate and buffer as described in the text and 0.00125 M cobalt. The experiments of Curves I_A and I_B contained no glycine; those of Curves II_A and II_B contained 0.0625 M glycine. Temperature 32.20°.

respectively, 52, 69, and 74 per cent. In the conjoint presence of magnesium and cobalt, the inhibition by glycine was only 26 per cent. The other results of Table IX show the same phenomenon, though to different degrees.

That cobalt can restore the capacity of magnesium to activate bone phosphatase inhibited by amino acid after action on the substrate has begun is demonstrated in the experiments described below and shown in Fig. 2. Two substrate-buffer mixtures were made up so that, after addition of 1 cc. of a bone phosphatase, RBR-d4, there would be a final reaction volume of 8 cc. and final concentrations of 0.024 M veronal buffer, 0.0125 M magnesium, and 0.0625 M glycine. The pH was optimal. Samples were taken 30 and 90 minutes after the beginning of the reaction. Within 30 seconds after the second sample had been taken, 0.1 cc. of 1 M cobalt and, in order to maintain the pH in the optimal range, 0.15 cc. of N sodium hydroxide were added to one reaction mixture; 0.25 cc. of distilled water was added to the second reaction mixture. Samples were taken 30 minutes, 60 minutes, and 120 minutes later. It may be seen from Curve II_A of Fig. 2 that the addition of cobalt led to an immediate increase in the reaction velocity which was much higher than that in the corresponding control hydrolysis containing only magnesium as the metal (Curve I_A).

TABLE IX

Influence of Conjoint Presence of Cobalt and Magnesium on Inhibition of Bone and Intestinal Phosphatase Activity by Amino Acids

The reaction conditions are as described in the text; temperature, 32.20°. Rat intestinal phosphatase, RIR-d1, and rat bone phosphatase, RBR-d2, were used.

Phosphatase preparation	Amino acid	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.				Activation due to		
		No metal added	0.0125 M Mg present	0.00125 M Co present	0.0125 M Mg + 0.00125 M Co present	Magnesium	Cobalt	Magnesium and cobalt
		γ	γ	γ	γ	*per cent*	*per cent*	*per cent*
Bone	None	0.19	0.73	0.61	0.85	280	220	340
	0.0625 M glycine	0.092	0.23	0.16	0.61	150	75	560
	0.156 " "	0.042	0.102	0.103	0.32	140	140	660
	0.125 " DL-alanine	0.080	0.24	0.14	0.58	200	60	540
	0.0125 " L-histidine	0.062	0.074	0.103	0.16	19	66	140
Intestinal	None	0.25	0.92	0.83	1.01	265	230	300
	0.156 M glycine	0.053	0.34	0.050	0.24	540	−5	350
	0.0375 M L-glutamic acid	0.11	0.45	0.11	0.39	310	0	260

A second pair of substrate-buffer mixtures was made up to contain cobalt and glycine. The extent of reaction was determined at 30 and 90 minutes. Immediately after the latter time, magnesium, to yield a final concentration of 0.0125 M, was added to the first reaction mixture while a corresponding volume of water was added to the second which served as a control. Curves I_B and II_B in Fig. 2 show that the same phenomenon, namely a marked increase in the reaction velocity, occurred upon the addition of magnesium. In other words, the conjoint presence of cobalt and magnesium was necessary in counteracting the inhibition of bone phosphatase by glycine.

Influence of Conjoint Presence of Cobalt and Magnesium on Inhibition of Intestinal Phosphatase Activity by Amino Acids—It has been shown that at inhibiting concentrations of amino acids the extent of activation of intestinal phosphatase by magnesium was the same as in the absence of amino acids, whereas the extent of activation by cobalt was reduced.

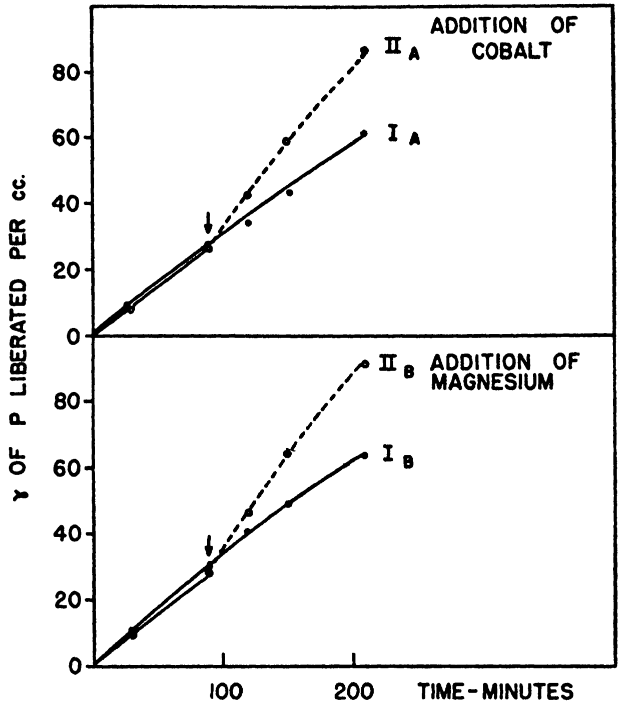

Fig. 2. The necessity for the conjoint presence of cobalt and magnesium in counteracting the inhibition of bone phosphatase by glycine. Curve I_A represents the course of the reaction of rat bone phosphatase, RBR-d4, in the presence of 0.0125 M magnesium and 0.0625 M glycine; Curve II_A is the same until 0.00125 M cobalt is added at 90 minutes (designated by arrow), whereupon there occurs an immediate increase in reaction rate as shown by the remaining, dashed portion of the curve. Curves I_B and II_B show, similarly, the increase in reaction rate resulting upon the addition of 0.0125 M magnesium to a reaction mixture containing 0.00125 M cobalt and 0.0625 M glycine. Temperature, 32.20°.

Table IX shows that in the presence of inhibiting concentrations of amino acids the degree of activation by a combination of cobalt and magnesium was greater than that due to cobalt alone but about the same as that due to magnesium alone. In other words, magnesium activated intestinal phosphatase to about the same extent whether amino acid or cobalt or both were present or absent. In contrast to the effect on bone phosphatase, cobalt did not affect the activating capacity of magnesium on intestinal phosphatase in the presence of inhibiting concentrations of amino acid.

Effect of Cyanide and Azide on Activation of Phosphatase by Cobalt—The observations that bone and intestinal phosphatases are inhibited very markedly by cyanide but are affected negligibly by azide gave rise to the possibility that cyanide forms a very slightly dissociated and azide a more dissociable complex with the metal component of the enzyme. It was of interest in this connection to see the extent to which cyanide and azide could counteract the activating effect of cobalt on phosphatase. Table X shows that in the presence of 0.00125 M cobalt 0.0125 M cyanide decreased the reaction velocity to a value below that obtained without cobalt or cyanide. In contrast, azide did not inhibit bone phosphatase either in the absence of cobalt or in the presence of 0.00125 M cobalt. Similar results were obtained when intestinal phosphatase, RIS-d, was used. These results show a parallelism between cobalt and the metal component of the enzyme in that both combine with cyanide to such an extent that phosphatase activity is greatly inhibited, whereas the extent of combination with azide is insufficient to affect the activity.

TABLE X

Effect of Cyanide and Azide on Activation of Phosphatase by Cobalt

The conditions of the reaction are as described in the text; temperature, 32.20°. Bone phosphatase, RBR-d4, and intestinal phosphatase, RIS-d, were used.

Inhibitor	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.			
	In bone phosphatase		In intestinal phosphatase	
	Without cobalt	With 0.00125 M cobalt	Without cobalt	With 0.00125 M cobalt
	γ	γ	γ	γ
None	0.31	0.65	3.75	7.19
0.0125 M sodium azide	*	0.66	3.57	7.03
0.0125 " cyanide	0.01	0.18	0.00	0.33

* This velocity was not determined here, since previously it had been shown that inhibition by this concentration of azide was negligible (Table VII).

Inhibition of Human Osteogenic Sarcoma Phosphatase by Amino Acids—It has been shown that an enzyme, originally characterized by its action on a particular substrate at a certain optimal pH and considered on the basis of such characterization to be identical in its properties regardless of the tissue in which it is found, may subsequently be proved to possess different inhibition characteristics which vary with the tissue source (1, 2, 27–29). Since human osteogenic sarcoma phosphatase represents an instance of rapid and excessive formation of an enzyme in a malignant process, it was of interest to compare the amino acid inhibition characteristics of this phosphatase with those of normal rat bone phosphatase. It was recognized that any differences which might be elicited would also have to be evaluated in terms of the species source.

Table XI summarizes the data previously reported for the inhibition of rat bone and intestinal phosphatases by amino acids and also includes new data on the inhibition by L-arginine. It was previously shown (1) that, whereas there was no significant difference between the bone and intestinal phosphatases with respect to inhibition by glycine or DL-alanine, the inhibition of bone phosphatase by basic amino acids was greater than that by the acidic acid, L-glutamic acid, and the inhibition of intestinal phosphatase by L-glutamic acid greater than that by the basic amino acids. Table XI shows that the extent of inhibition of human osteogenic sarcoma phosphatase by the various amino acids appears, within experimental variation, to be the same as that of normal rat bone phosphatase.

TABLE XI

Comparison of Inhibition of Rat Intestinal, Rat Bone, and Human Osteogenic Sarcoma Phosphatases by Amino Acids

The values in the second and third columns, with the exception of those for L-arginine, are from an earlier paper (1).

Amino acid	Concentration of amino acid required for 50 per cent inhibition of		
	Rat intestinal phosphatase	Rat bone phosphatase	Human osteogenic sarcoma phosphatase
	M	M	M
Glycine	0.055	0.040	0.035
DL-Alanine	0.138	0.096	0.102
L-Glutamic acid	0.030	0.105	0.117
L-Arginine	0.128	0.010	0.010
L-Histidine	0.006	0.003	0.002

Several rat bone and intestinal phosphatases, two different sarcoma preparations, MOSA-d1 and MOSB-d1, and the purified preparation, MOSB-P, were used in the above experiments. The concentration of magnesium was 0.0125 M in all experiments.

Influence of Magnesium on Inhibition of Human Osteogenic Sarcoma Phosphatase by Amino Acids and by Cyanide—Table XII shows that the extent of activation of human osteogenic sarcoma phosphatase by magnesium is decreased in the presence of an inhibiting concentration of amino acid or cyanide and indeed that the presence of magnesium causes an additional depressant effect. Similar results were obtained with the purified preparation, MOSB-P. In this respect, therefore, human osteogenic sarcoma also resembles normal rat bone phosphatase.

TABLE XII

Influence of Magnesium on Inhibition of Human Osteogenic Sarcoma Phosphatase by Amino Acids and by Cyanide

The conditions of the reaction are as described in the text; temperature, 32.20°.

Phosphatase preparation	Inhibitor	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.		Activation
		No added magnesium	0.0125 M magnesium present	
		γ	γ	*per cent*
Human osteogenic sarcoma, MOSB-d1	None	9.95	15.6	59
	0.00125 M cyanide	5.50	3.66	−33
	0.0125 " L-histidine	4.94	2.51	−49
Purified human osteogenic sarcoma, MOSB-P	None	2.02	3.76	86
	0.00125 M cyanide	0.90	0.68	−24
	0.0125 " L-histidine	0.64	0.44	−31
	0.50 M L-aspartic acid	0.51	0.46	−10

TABLE XIII

Influence of Magnesium, of Cobalt, and of Magnesium and Cobalt Present Conjointly on Inhibition of Human Osteogenic Sarcoma Phosphatase by Amino Acids

The conditions of the reaction are as described in the text; temperature, 32.20°. Human osteogenic sarcoma phosphatase, MOSB-d2, was used.

Amino acid	Reaction velocity, as P liberated as inorganic phosphate per cc. per min.				Activation due to		
	No metal added	0.0125 M magnesium present	0.00125 M cobalt present	0.0125 M magnesium, 0.00125 M cobalt present	Magnesium	Cobalt	Magnesium and cobalt
	γ	γ	γ	γ	*per cent*	*per cent*	*per cent*
None	5.51	7.11	7.23	6.90	29	31	25
0.125 M glycine	1.84	1.18	2.33	3.40	−36	27	65
0.0125 M L-histidine	1.40	0.88	1.55	1.83	−37	11	31

Influence of Conjoint Presence of Cobalt and Magnesium on Inhibition of Human Osteogenic Sarcoma Phosphatase by Amino Acids—Table XIII shows that cobalt and magnesium, either alone or present together, activate human osteogenic sarcoma phosphatase. In the presence of inhibiting concentrations of glycine or L-histidine, the activating effect of cobalt alone was decreased slightly, and, as will be recalled from Table XII, the activating effect of magnesium was markedly decreased and even reversed.

However, when cobalt and magnesium were present conjointly at these concentrations of amino acids, the activating effect of magnesium was restored.

Fig. 3 demonstrates that cobalt can restore the capacity of magnesium to activate osteogenic sarcoma phosphatase inhibited by amino acid, even after action on the substrate has begun. The experiments were conducted in essentially the same manner as those listed in Fig. 2. The first mixture contained cobalt and glycine to yield final concentrations of 0.00125 M and 0.0625 M, respectively. The second and third reaction mixtures contained magnesium and glycine to yield final concentrations of 0.0125 M and 0.0625 M, respectively, and the fourth reaction mixture contained cobalt and magnesium in the corresponding concentrations, but no glycine. Samples were taken 30 minutes after the beginning of the reaction. Cobalt and, to maintain optimal pH under the new conditions, sodium hydroxide were added to the third reaction mixture immediately after sampling. Suitable adjustments for volume were made in the other reaction mixtures. It may be seen from Fig. 3 that, following the introduction of cobalt in a final concentration of 0.00125 M into the third reaction mixture, there was an immediate increase in the reaction velocity. This velocity became much greater than in the first control mixture which contained only magnesium

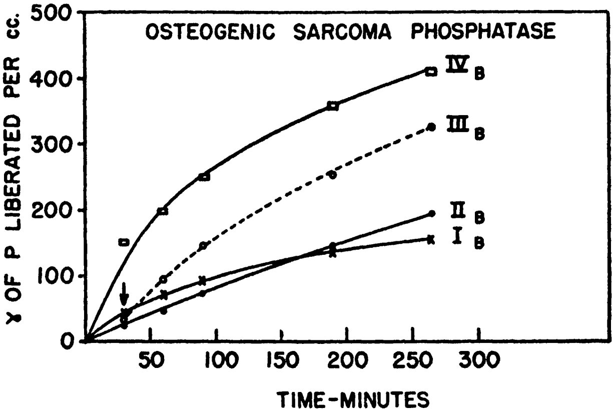

FIG. 3. The necessity for the conjoint presence of cobalt and magnesium in counteracting the inhibition of osteogenic sarcoma phosphatase by glycine. Curve $\mathrm{I_B}$ represents the course of the reaction in the presence of 0.00125 M cobalt and 0.0625 M glycine; Curve $\mathrm{II_B}$ is the course in the presence of 0.00125 M magnesium and 0.0625 M glycine. Curve $\mathrm{III_B}$ is the same as Curve $\mathrm{II_B}$ until 0.00125 M cobalt is added at 30 minutes, as shown by the arrow; the rest of Curve $\mathrm{III_B}$, shows a marked increase in the reaction velocity after the addition of cobalt. Curve $\mathrm{IV_B}$ represents the course in the presence of both cobalt and magnesium but in the absence of glycine. Temperature, 32.20°.

in addition to the glycine or in the second control mixture which contained only cobalt in addition to the amino acid. Indeed the introduction of cobalt into the third reaction mixture, in conjunction with the magnesium already present, so increased the reaction velocity of the glycine-inhibited phosphatase that it approached the velocity in the fourth reaction mixture which contained cobalt and magnesium in corresponding concentrations, but no glycine.

Effect of Sodium Azide on Human Osteogenic Sarcoma Phosphatase—Sodium azide was found to exert a negligible inhibitory effect on human osteogenic sarcoma phosphatase, preparation MOSB-d1, either in the absence or presence of magnesium. Thus, at concentrations of 0, 0.0025 M, 0.025 M, and 0.250 M sodium azide, the reaction velocities were respectively 15.6, 16.5, 17.1, 12.1 γ of P per cc. per minute when 0.0125 M magnesium was present, and 9.8, 12.6, 12.2, 9.1 γ of P per cc. per minute in the absence of magnesium.

Influence of Cobalt on pH Optimum of Human Osteogenic Sarcoma Phosphatase in Presence of Amino Acids—It was found that, when cobalt or cobalt and magnesium were present, the pH optimum of the osteogenic sarcoma phosphatase, MOSB-d2, inhibited by glycine was shifted towards the alkaline side to a value of about 10.5. Magnesium alone did not cause such a shift in the pH optimum of the amino acid-inhibited phosphatase. In the absence of inhibiting amino acids, the pH optimum was, as usual, between 9.0 and 9.5, whether magnesium or cobalt or a combination of these metals was present. In these respects, therefore, human osteogenic sarcoma phosphatase also resembled normal rat bone phosphatase.

DISCUSSION

In the present study it has been shown that in the conjoint presence of cobalt and magnesium, achieved either before or after the beginning of the reaction, the degree of activation of bone or osteogenic sarcoma phosphatase which had been inhibited by amino acid was much greater than with either cobalt or magnesium alone and counteracted, to varying degrees, this inhibition. In contrast, the degree of activation of amino acid-inhibited intestinal phosphatase was about the same in the conjoint presence of cobalt and magnesium as in the presence of magnesium alone. The formulation consistent with these findings is that bone phosphatase requires the combined presence of magnesium and at least one other heavy or transitional metal ion for activity, that coordinative combination of this second metal with cyanide or amino acid results in decreased enzyme activity and failure of activation by magnesium, that activation of bone phosphatase by magnesium is mediated through a transitional or heavy metal ion, and that restoration of the activity which has been inhibited by amino acid may be brought about by the addition to the substrate-enzyme system of a transitional metal, cobalt, which is presumably similar to, if not the same as, the metal component of the enzyme. In contrast, although the intestinal phosphatase also contains a metal component essential for its activity, activation by magnesium is independent of whether this metal is combined with amino acid; in other words, activation of intestinal phosphatase by magnesium is not mediated through this metal component.

The way in which cobalt may restore the activation by magnesium requires further consideration. Burk and his coworkers (14–16) have recently shown that cobalt reacts with histidine and other amino acids to form cobaltous-amino acid complexes which interact reversibly with oxygen gas to form oxygenated compounds and are then transformed into irreversible oxidation compounds. There are several lines of evidence to show that, under the conditions of our experiments, the extent of the irreversible oxidation of amino acids by cobalt is so small as to play only a negligible rôle in counteracting the inhibitory effect. If degradation of amino acids occurred to any appreciable extent, then the rate of hydrolysis in a reaction mixture containing cobalt and amino acid should be accelerating constantly. As may be seen from Figs. 2 and 3, this is not the case. Secondly, the time-action curve is essentially of the same form whether magnesium or cobalt is present together with the amino acid. Finally and most directly, Burk[2] has estimated that a relatively small amount of oxygenation products would be formed in a reaction mixture containing 0.06 to 0.12 M glycine and 0.001 M cobalt during 30 to 60 minutes at 30° and a pH level of 10 to 11.

Nor does it seem likely that the action of cobalt in mediating the activation by magnesium of amino acid-inhibited bone or osteogenic sarcoma phosphatases is due merely to the removal of the amino acid from the field of reaction through the formation of a reversible cobaltous-amino acid complex. For such an assumption would not explain the immediate and marked acceleration in velocity which resulted when magnesium was added 90 minutes after the beginning of a reaction in which cobalt and glycine had been present from the start.

Our findings are more in accord with the formulation that cobalt either replaces the essential enzyme metal component which has been bound by the amino acid, or that it displaces this metal component from its combination with the amino acid, and that cobalt or this essential metal, as part of the enzyme, then mediates the activation by magnesium. It may be readily appreciated that the extent to which cobalt, acting conjointly with magnesium, can restore the activity of bone or osteogenic phosphatase which has been inhibited by a particular amino acid would depend upon the relative magnitudes of the dissociation constants of the essential metal-enzyme linkage, the essential metal-amino acid complex, the cobalt-enzyme complex, the reversible cobaltous-amino acid complex, the reversible oxygenated cobaltous-amino acid complex, and the velocity constants for the formation of the various phases of the irreversible oxidation reactions.

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[2] Burk, D., personal communication.

Although the above formulations appear well supported by our data, it is necessary to discuss briefly, if only to dismiss, another possible explanation for the failure of magnesium to activate bone or osteogenic tumor phosphatases at high concentrations of cyanide or amino acid. It may be assumed that a preparation of bone or of osteogenic sarcoma phosphatase is a mixture of two enzyme components, one of which is inhibited by cyanide or amino acid and is activatable by magnesium, while the other component is not inhibited by cyanide or amino acid and is not activated by magnesium.[3] However, a more purified preparation of osteogenic sarcoma phosphatase, MOSB-P, behaved in the same manner as the rat bone preparations with regard to inhibition by amino acids and cyanide. The assumption of the existence of two phosphatase components was also contradicted by the findings that a sufficiently high concentration of histidine (0.125 M) or of cyanide (0.0125 M) inhibited practically completely (96 to 99 per cent) the activity of bone phosphatase both in the presence and absence of magnesium.

SUMMARY

1. The degree of activation of bone phosphatase by magnesium was decreased and indeed in some instances transformed into a retardant effect at inhibiting concentrations of amino acid or cyanide, whereas the degree of activation of intestinal phosphatase was unaffected. The extent of activation of bone phosphatase by cobalt was decreased slightly and that of intestinal phosphatase markedly as the concentration of amino acid was increased.

2. In the conjoint presence of cobalt and magnesium, the degree of activation of bone phosphatase which had been inhibited by amino acids was much greater than with either cobalt or magnesium alone and tended to counteract the inhibition by amino acids. The addition of cobalt to a reaction mixture in which bone phosphatase was hydrolyzing sodium β-

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[3] This assumption is quite different from that made by Cloetens (30) in his classification of alkaline phosphatases. According to this author phosphatase I, found in considerable proportions in the liver, is accelerated 50 to 100 times by an optimal concentration of magnesium and is not inhibited by cyanide in the presence of magnesium. Phosphatase II, present in relatively large amounts in the bone and intestine, is accelerated only slightly by magnesium and inhibited considerably by cyanide.

glycerophosphate in the presence of magnesium and inhibiting amino acid, or the addition of magnesium to a reaction mixture containing cobalt and inhibiting amino acid, led to an immediate acceleration in the rate of reaction.

3. The degree of activation of intestinal phosphatase which had been inhibited by amino acid was the same in the conjoint presence of cobalt and magnesium as in the presence of magnesium alone.

4. Sodium azide had a negligible inhibitory effect on the activity of bone and intestinal phosphatases and, in contrast to cyanide, did not counteract the activation due to cobalt.

5. In the presence of cobalt, an inhibitory concentration of amino acid led to a shift in the optimal pH zone for bone phosphatase, but not for intestinal phosphatase, towards the alkaline side.

6. Human osteogenic sarcoma phosphatase resembled normal rat bone phosphatase with respect to (*a*) the extent of inhibition by various amino acids, (*b*) the decrease of activating effect of magnesium or even its reversal in the presence of inhibiting concentrations of amino acids or cyanide, (*c*) the shift of the pH optimum to the alkaline side in the presence of cobalt and amino acid, (*d*) the necessity for the conjoint presence of cobalt and magnesium in counteracting inhibition by amino acids.

7. The above findings were interpreted to indicate that amino acids inhibit bone, intestinal, and osteogenic sarcoma phosphatases by combination with an essential heavy or transitional metal component of these enzymes and that this component mediates the activation of bone and osteogenic sarcoma phosphatases, but not of intestinal phosphatase, by magnesium.

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THE METABOLISM OF ARTERIAL TISSUE

I. RESPIRATION OF RAT THORACIC AORTA*

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Although aging is not without some influence upon many tissues, the degenerative changes that occur in the artery with advancing years are unique in their import. The characteristic lesion found in large arteries, such as the aorta of man, is the atheroma which begins as a lipide plaque, confined at first to the intima but later extending to the deeper layers of this structure. An explanation as to why the artery lends itself to this peculiar type of degeneration has not as yet been forthcoming. A study of the metabolic characteristics of arterial tissues was, therefore, undertaken. The present report deals with the respiration of the aorta of rats of two age groups, namely 1 to 2 months and 14 to 18 months. The response of this tissue to induced hyperthyroidism and hypothyroidism has also been investigated.

EXPERIMENTAL

Rats of the Long-Evans strain were sacrificed by fracture of their cervical vertebrae. The thoracic aorta and a portion of the main lobe of the liver were rapidly removed and placed in an oxygenated Ringer-phosphate solution buffered to pH 7.4 (1). The artery was freed of adherent fat and connective tissue and then cut along its longitudinal axis so as to provide a thin sheet of tissue. Liver slices were prepared free-hand with a thin razor blade. The tissues were blotted on moist filter paper, weighed, and transferred to Warburg flasks containing 3 cc. of the Ringer-phosphate solution. Oxygen consumption was determined by the direct method of Warburg (2). The flasks were placed in the constant temperature bath maintained at 38° and the flask flushed with oxygen for 3 to 5 minutes. 15 minutes were allowed for temperature equilibration. Manometer readings were made at 15 minute intervals.

The amount of fresh artery employed in each determination of O_2 consumption was about 100 mg., although amounts as low as 75 mg. and as high as 150 mg. were sometimes used. The whole thoracic aorta of an

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adult rat provided sufficient material for a single determination. Since the thoracic aorta of the 1 to 2 month-old rat weighed less than 50 mg., it was necessary to pool the aortas of two or three such rats for a single determination.

The O_2 consumption of the arteries was related to their nitrogen content as well as to their initial dry weights. Nitrogen was determined on the entire contents of the Warburg flasks at the end of the period of respiration. The dry weight (initial) to nitrogen ratios were determined for forty samples of fresh artery and found to be 7.49 ± 0.52 (standard deviation) for both age groups and this value was used in the calculation of Q_{O_2}, *i.e.* c.mm. of O_2 consumed per hour per mg. of dry tissue in an atmosphere of oxygen. The thickness of the arterial sheet did not appear to be a limiting factor in its oxygen uptake.

TABLE I

Oxygen Consumption of Vascular Tissues of Rat

Ringer-phosphate buffer containing 0.2 per cent glucose; pH 7.4; O_2 as gas phase; temperature 38°.

Experiment No.*	Tissue	$Q^{O_2}_{O_2}$ (N)†
1	Thoracic aorta	8.1
	Abdominal aorta	6.2
	Inferior vena cava	5.3
2	Thoracic aorta	8.3
	Abdominal aorta	7.8
	Inferior vena cava	5.1

* The vascular tissues in each experiment were obtained from a single rat 14 months of age, weighing 400 gm.

† Oxygen consumption in c.mm. of the tissue in pure oxygen atmosphere per hour per mg. of tissue nitrogen (2).

Results

The oxygen consumption of two portions of the aorta (thoracic and abdominal) and of the inferior vena cava is recorded in Table I. The tissues obtained from a single rat were compared in each experiment.

The values for the $Q^{O_2}_{O_2}$ (N)[1] of thoracic aorta were 8.1 in Experiment 1 and 8.3 in Experiment 2. The values for the abdominal aorta were only slightly lower (6.2 and 7.8, respectively). It should not be inferred, however, that a significant difference exists in the respiratory rates of these two portions of the aorta, because the presence of numerous bifurcations in the abdominal portion made it difficult to excise and free it of all extraneous tissue.

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[1] The expression $Q^{O_2}_{O_2}$ (N) has been employed to indicate the c.mm. of O_2 consumed per hour per mg. of tissue nitrogen in an atmosphere of oxygen (2).

The $Q_{O_2}^{O_2}$ (N) of the inferior vena cava was lower than that of either portion of the aorta. This finding is consistent with the histological differences that exist between these two types of vascular tissue. Arteries, because of their relatively thick muscular walls, might be expected to possess a higher metabolic rate than veins whose walls consist largely of fibrous tissue.

In all experiments recorded below the thoracic portion of the aorta was used because of the ease with which it can be removed and freed of adherent fat and connective tissue.

TABLE II

Comparison of the Oxygen Consumption of Arterial and Hepatic Tissues of Rats 1 to 2 Months and 14 to 18 Months Old

Ringer-phosphate buffer containing 0.2 per cent glucose; pH 7.4; O_2 as gas phase; temperature 38°.

	Thoracic aorta		Liver	
Age of rat	1-2 mos.	14-18 mos.	1-2 mos.	14-18 mos.
No. of determinations	17	24	10	16
Average Q_{O_2}[*]	1.06 ± 0.14[†]	1.09 ± 0.19	9.7 ± 0.74	8.4 ± 1.66
" $Q_{O_2}^{O_2}$ (N)	7.8 ± 1.03	8.2 ± 1.43	53.4 ± 4.6	47.1 ± 9.3

[*] Based on the initial dry weight.

[†] Standard deviation.

The Q_{O_2} values for the thoracic aortas of young rats did not differ significantly from those of rats 14 to 18 months old (Table II).

In Fig. 1, the rate of respiration of the artery in a Ringer-phosphate buffer is shown to remain constant for periods as long as 3 hours. This was true for rats of both age groups (1 to 2 months and 14 to 18 months old) when respiration measurements were made on sheets of arterial tissue. A constancy in the oxygen uptake of tissue slices for such long periods has been interpreted to indicate that the cell structure remains intact under the conditions studied and that no loss by diffusion or destruction of essential cell elements has occurred (3). This is borne out by the finding that destruction of the cellular organization of the artery by homogenization markedly reduced its oxygen uptake (Fig. 1).

Effect of Hyperthyroidism and Hypothyroidism on Respiration of Rat Aorta—The relation of thyroid activity to the respiratory rate of the rat aorta is shown in Table III. Hyperthyroidism was induced in rats by

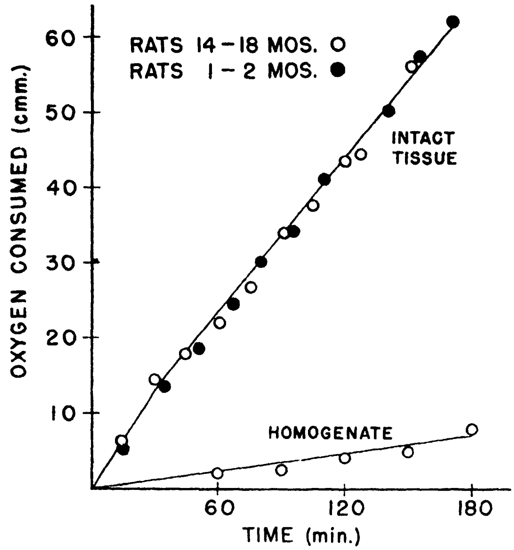

FIG. 1. Respiration of sheets of thoracic aorta and homogenate. Oxygen uptake in c.mm. per 100 mg. of initial weight of tissue.

TABLE III

Respiration of Arteries and Livers of Rats Fed Thyroid and Propylthiouracil

Ringer-phosphate buffer containing 0.2 per cent glucose; pH 7.4; O_2 as gas phase; temperature 38°.

Diet		Rats, 1-2 mos., 50-150 gm.		Rats, 14-18 mos., 350-450 gm.	
		Artery	Liver	Artery	Liver
Normal	Q_{O_2}*	1.06 ± 0.14†	9.7 ± 0.74	1.09 ± 0.19	8.4 ± 1.66
	Determinations	17	10	24	16
Fed 0.2% thyroid 10 days	Q_{O_2}	1.32 ± 0.22	12.7 ± 1.12	1.48 ± 0.28	10.3 ± 1.17
	Determinations	16	18	24	14
	% increase	25	31	38	23
Fed 0.2% propylthiouracil 30 days	Q_{O_2}	0.86 ± 0.19	7.0 ± 1.13	0.79 ± 0.15	6.2 ± 1.27
	Determinations	22	16	17	21
	% decrease	19	28	27	27

* Based on the initial dry weight.
† Standard deviation.

feeding them for 10 days the stock diet[2] to which had been added 0.2 per cent desiccated thyroid. It is now well established that goitrogenic substances depress the metabolism of rats by interfering with the formation of thyroxine (4). The feeding of a goitrogen was therefore employed to induce hypothyroidism. Rats were fed, for 30 days, the stock diet to which had been added 0.2 per cent propylthiouracil.

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[2] The stock diet consisted of whole wheat 35 per cent, casein 36 per cent, whole milk powder 8 per cent, alfalfa meal 5 per cent, fish meal 5 per cent, lard 2.5 per cent, fish oil 2.5 per cent, and salt 6 per cent.

Rat aorta resembles tissues like liver and kidney in its response to thyroid stimulation (5–7) and to depressed thyroid activity (8). The Q_{O_2} values for aortas excised from rats fed the desiccated thyroid were 25 to 38 per cent higher than those obtained from normal rats and these increases were about the same as those observed for liver slices (Table III).

The extent to which the feeding of propylthiouracil reduced the Q_{O_2} values in both groups of rats (young and mature) was quite similar for artery and liver (Table III).

TABLE IV

Effect of Oxidizable Substances on O_2 Uptake in Arterial Tissue

Ringer-phosphate buffer; pH 7.4; O_2 as gas phase; temperature 38°. The substances were added after a 1 hour control period. The figures express comparative rates when the O_2 uptake of tissue alone is taken as 100. An amount of a substrate was added to yield a concentration of 0.02 M in the bath. All values recorded below are the averages of three to five closely agreeing results.

Substrates	Rats 1-2 mos. of age	Rats 14-18 mos. of age
Succinate	244	215
Lactate	144	132
Pyruvate	135	124
α-Ketoglutarate	129	128
Citrate	127	127
Acetate	126	116
Glutamate	119	113
Glycine	99	98
Glucose	95	95
Alanine	92	100

Effects of Oxidizable Substances on Oxygen Consumption of Rat Aorta—Since the respiration of rat aorta remains constant for periods as long as 3 hours (Fig. 1), the substances to be tested were added to the medium after a 1 hour control measurement had been made. The control rate of respiration of each sample was determined during the 1st hour, and at the end of this period the substance to be tested was added to the medium from the side arm of the Warburg flask, and the respiratory measurements continued for a 2nd hour. In Table IV, the Q_{O_2} values for initial control periods are compared with those observed after the addition of various substrates.

0.2 M solutions of each of the substrates tested (Table IV) were prepared and adjusted to pH 7.4. 0.3 cc. of a solution was placed in the side arm and 2.7 cc. of Ringer-phosphate buffer were pipetted into the main compartment of the Warburg flask. Thus, after tilting the manometer so as to transfer the contents of the side arm to the main compartment, the final concentration of the substrate in the medium was 0.02 M.

Arterial tissue oxidized succinate at a rate higher than that of any of the other substrates examined; the increase in O_2 consumption was about equal to that produced by succinate on kidney and adipose tissue (9).

Lactate, pyruvate, and acetate also stimulated oxygen consumption of arterial tissue. The presence of these substrates increased the Q_{O_2} of thoracic aorta by 20 to 40 per cent and, in this respect, the thoracic aorta again resembles a tissue like kidney (10). Evidence for the utilization of acetate by rat thoracic aorta is presented in Paper II (11).

α-Ketoglutarate and citrate[3] produced a 30 per cent increase in the rate of respiration of arterial tissue; the addition of glutamate increased the Q_{O_2} to the extent of 15 per cent.

TABLE V

Effect of Inhibitors on O_2 Uptake in Arterial Tissue

Ringer-phosphate buffer; pH 7.4; O_2 as gas phase; temperature 38°. The substances were added after a 1 hour control period. The figures express comparative rates when the O_2 uptake of tissue alone is taken as 100. All substances were added to give a 0.02 M concentration in the tissue bath. All values recorded below are the averages of three to five closely agreeing results.

Inhibitor	Rats 1-2 mos. of age	Rats 14-18 mos. of age
Malonate	93	88
Fluoride	65	56
Azide	54	52
Iodoacetate	32	34

The addition of glucose or glycine or alanine failed to increase the oxygen uptake of the rat aorta. This does not imply that these substances are not utilized. Barron has shown that, despite the failure of certain substrates to increase the respiratory rate, they may nevertheless be utilized at appreciable rates (12).

Effects of Inhibitors on Respiration of Arterial Tissue—The enzyme inhibitors, malonate, fluoride, azide, and iodoacetate, were added to the Ringer-phosphate medium after a control measurement (1 hour) had been made as described in the preceding section. The concentration of an inhibitor in the medium was 0.02 M. The results are recorded in Table V.

The most pronounced inhibition of respiration was found in the presence of iodoacetate. Azide and fluoride depressed the respiration of rat thoracic aorta by 30 to 50 per cent. Differences in the action of these inhibitors upon the respiration of the arteries of young and old animals were not observed.

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[3] The effect of citrate was determined in a calcium-free medium. The absence of calcium did not change the Q_{O_2} of the artery.

0.02 M malonate failed to produce a marked inhibition in the oxygen consumption of arterial tissue. Seven experiments (four on young rats and three on mature rats) were performed to test the action of this substance. In two experiments, no decrease in respiration was observed, and in no case was the respiration depressed by more than 13 per cent. The malonate employed in this study was also tested on liver slices; in the presence of 0.02 M malonate, the oxygen consumption of this tissue was decreased 45 per cent.

The failure of malonate to inhibit respiration of rat thoracic aorta is somewhat surprising because of the pronounced stimulation in oxidative metabolism observed (Table IV) in the presence of succinate. In this connection it is of interest to note that Gordon and Heming have reported that malonate does not depress the rate of respiration of *normal* rat diaphragm but does when the Q_{O_2} of this tissue is raised by the feeding of desiccated thyroid (5).

TABLE VI

Effects of Malonate and Succinate on Respiration of Thoracic Aorta of Rat

Ringer-phosphate buffer; pH 7.4; O_2 as gas phase; temperature 38°.

Experiment No.	Substrate in bath during 1st hr., 0.02 M	Substrate added to bath after 1 hr. of incubation, 0.02 M final concentration	Q_{O_2}*	
			1st hr.	2nd hr.
1	Succinate	Malonate	2.2	1.3
2	"	"	2.2	1.1
3	"	"	3.5	1.4
4	"	"	1.5	0.6
5	Malonate	Succinate	0.8	1.6
6	"	"	0.8	1.8

* Based on the initial dry weight.

In order to determine whether malonate action on the succinic dehydrogenase system of the rat artery was masked by the low respiratory rate of artery the following experiments were performed.

In Experiments 1 to 4, arterial tissue from mature rats was placed in Warburg flasks which contained, in the main compartment, 2.7 cc. of 0.02 M succinate in Ringer-phosphate and, in the side arm, 0.3 cc. of 0.2 M malonate. After the rate of respiration of the artery for 1 hour in the presence of succinate was determined, the malonate was added to the medium and the determinations continued for an additional hour. Under these conditions malonate inhibited the heightened O_2 consumption of the artery by 40 to 60 per cent (Table VI).

In Experiments 5 and 6, 0.02 M malonate was placed directly in the medium and 0.2 M succinate in the side arm. The transfer of succinate from the side arm to the malonate-containing medium doubled the rate of respiration of the arterial tissue (Table VI).

The results presented in Table VI suggest that, despite the failure of malonate to depress the respiratory rate of arterial tissue excised from the *normal* rat, the succinic dehydrogenase system does participate in the metabolism of this tissue.

DISCUSSION

In assessing the significance of the slice technique, Potter has aptly stated that the oxygen uptake of a tissue slice is the resultant of a large number of competing and cooperating enzyme systems (13). The rate of this over-all activity in rat thoracic aorta, as judged by the Q_{O_2} values presented here, is slow, approximately one-tenth that of liver and about one-twentieth that of kidney.

Enzyme inhibitors, particularly if selective, have provided useful information on the intermediary metabolism of tissues. The interpretation of their action has been discussed by Cohen (14) and by Krebs (15). Thus the response in the respiration of rat thoracic aorta to malonate and succinate, shown in Tables V and VI, indicates that succinic dehydrogenase participates in this process, whereas the results obtained with fluoride merely suggest that phosphorylated intermediates are also involved (16). These findings, in addition to the oxidative response of this tissue to pyruvate and to some members of the tricarboxylic acid cycle, provide presumptive evidence that carbohydrate is utilized by the rat arterial tissue.

The oxygen uptake by thoracic aorta excised from rats, in which hyperthyroidism and hypothyroidism had been induced, shows that, like kidney, liver, and muscle, arterial tissue possesses oxidative systems that respond to thyroxine. This observation is of particular interest in connection with the reports that the administration of desiccated thyroid prevents atheromatosis in rabbits fed cholesterol (17–19).

SUMMARY

1. The respiratory characteristics of arterial tissue of the rat have been investigated. The Q_{O_2} of rat thoracic aorta was about 1 and the $Q_{O_2}^{O_2}$ (N) about 8. Arterial tissue of the rat thus respires at a rate about one-tenth that of liver slices prepared from the same animal.

2. No marked difference was observed in the oxygen consumption of thoracic and abdominal portions of the aorta. The $Q_{O_2}^{O_2}$ (N) of the inferior vena cava was lower than that of the aorta.

3. No significant difference in oxidative metabolism of aorta excised from rats 1 to 2 months and rats 14 to 18 months old was demonstrated.

4. Arterial tissue oxidized succinate, lactate, pyruvate, α-ketoglutarate, citrate, acetate, and glutamate. Glycine, glucose, and alanine did not increase the rate of respiration of the aorta. Succinate produced the most pronounced acceleration.

5. The oxygen consumption of arterial tissue was inhibited by fluoride, azide, and iodoacetate. Malonate produced no appreciable inhibition. Malonate did, however, depress respiration of rat thoracic aorta when its oxidative metabolism had been stimulated by succinate.

6. The Q_{O_2} of rat thoracic aorta was dependent upon the state of thyroid activity; it was increased by feeding rats desiccated thyroid and decreased by feeding them propylthiouracil.

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THE METABOLISM OF ARTERIAL TISSUE

II. LIPIDE SYNTHESES: THE FORMATION IN VITRO OF FATTY ACIDS AND PHOSPHOLIPIDES BY RAT ARTERY WITH C^{14} AND P^{32} AS INDICATORS*

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The increase in the lipide content of the artery is one of the significant changes that occur in this tissue with aging. Moreover, atherosclerosis, the commonest form of arterial degeneration, is also largely a lipide metamorphosis. In the advanced arteriosclerotic lesion, a complete replacement of normal arterial structure by lipides and minerals has occurred (1). The fact that a sustained hyperlipemia, induced either by cholesterol feeding in rabbits (2) and birds (3) or by stilbestrol injection in birds (4), produces atherosclerosis has led to the view that the accumulated lipides in the aging or degenerated artery represent an infiltration from plasma (1). But the increased lipide content of the artery that occurs *in man* with age is neither preceded nor accompanied by a rise in the level of plasma lipides. Thus Page *et al.* demonstrated that, in normal men, the amount and composition of lipides in plasma do not change between the ages of 20 and 90 years (5). The most satisfactory evidence on this point has been provided by Landé and Sperry who compared the degree of atherosclerosis of the aorta with the concentration of serum cholesterol in 123 subjects who died suddenly from violence (6). They concluded that the severity of atherosclerosis in man is not dependent upon the level of serum cholesterol. It would thus appear that factors other than, or in addition to, infiltration of plasma lipides play a part in the formation of atherosclerosis.

A study of the metabolism of arterial tissue was undertaken in order to throw additional light on the intraarterial factors that play a part in the development of arteriosclerosis. In the present investigation, the capacity of rat artery to synthesize lipides has been studied with radioactive acetate (fatty acid synthesis) and radioactive phosphate (phospholipide formation).

Synthesis of Fatty Acids from $C^{14}H_3C^{14}OONa$

The arteries were removed and prepared as described in Paper I (7). To obtain the necessary amount of arterial tissue, thirty-five rats were

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sacrificed in Experiments 1 and 2 and 80 in Experiment 3. 500 mg. portions of aorta were placed in each of several 50 ml. Erlenmeyer flasks. The medium in each flask consisted of 5.0 cc. of a bicarbonate-Ringer's solution to which had been added sodium acetate[1] labeled at both of its carbons with C^{14}. At the end of a 3 hour incubation period at 37.5°, the contents of the several flasks used in each experiment were combined and their fatty acids and cholesterol extracted.

The results of Experiments 1 to 3 are recorded in Table I. In 3 hours from 0.4 to 0.7 per cent of the labeled acetate had been converted to fatty acids.

Table I

Conversion of $C^{14}H_3C^{14}OONa$ to Fatty Acids by Rat Thoracic Aortas

Experiment No.	Thoracic aortas		Total C^{14} added	Recovery of C^{14} in					Average equivalent weights of fatty acids isolated
				Aqueous fraction		Fatty acid fraction			
	Amount	Total fatty acids		Total counts per min.	Per cent of added counts	Total counts per min.	Per cent of added counts	Specific activity*	
	gm.	*per cent wet weight*	*counts per min.*						
1	2.5 (35)†	6.8	6.3×10^6‡	2.6×10^6	41.5	4.5×10^4	0.71	265	280
2	2.8 (35)	5.0	6.3×10^6	3.2×10^6	51.4	2.6×10^4	0.41	184	275
3	6.2 (80)	4.2	1.5×10^7	4.9×10^6	32.3	8.3×10^4	0.55	320	294

* Counts per minute per mg. of fatty acids.

† The figures in parentheses refer to the number of rats sacrificed to obtain the gm. of thoracic aorta indicated.

‡ The specific activity of $C^{14}H_3C^{14}OONa$ used was 1.6×10^6 counts per minute per mg.

The conversion of acetate to fatty acids by rat liver slices was studied under similar conditions. A total of about 11 gm. of rat liver slices was incubated in the presence of $C^{14}H_3C^{14}OONa$ and analyzed for fatty acids. The results of this experiment are recorded in Table II. About 5 per cent of the added C^{14} was recovered in the fatty acid fraction.

Since the isolation of the radioactive fatty acids from artery and liver was carried out in the presence of a large fraction of the radioactive acetate that had been added to the medium, it became necessary to determine the extent to which the latter was carried over into the fatty acid fraction during its extraction. This was done in the following manner. 10 gm. of rat liver were digested for 3 hours in alcoholic KOH. 3.1 mg. of C^{14}-labeled sodium acetate with an activity of 5 million counts per minute were then added to the digest, and its fatty acids isolated. This amount of activity was greater than that encountered in the aqueous fraction of the artery experiment (Table I). The amounts of radioactivity recovered in the fatty acids isolated from the liver digests are recorded under Experiments 2 and 3 in Table II. Only 0.08 and 0.09 per cent of the added acetate was recovered in the fatty acid fractions.

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[1] We are indebted to Dr. H. A. Barker for the doubly labeled acetate used in this investigation. It was prepared from $C^{14}O_2$ with the aid of *Clostridium thermoaceticum* (8).

Cholesterol was also isolated as the digitonide from the arterial tissue and its radioactivity determined. Even though large numbers of rats (as many as 80 in Experiment 3) were sacrificed, the amounts of cholesterol isolated were insufficient, unfortunately, to permit an accurate determination of the radioactivity of cholesterol and its derivatives.

TABLE II

Conversion of $C^{14}H_3C^{14}OONa$ to Fatty Acids by Liver Preparations

Experiment No.	Tissue used	Total C^{14} added	Recovery of C^{14} in fatty acids		
			Total counts	Per cent of added counts	Specific activity*
	gm.				
1. Liver slice	10.6	2.3×10^6†	1.2×10^5	5.2	379
2. Digested liver‡	10.0	5.0×10^6§	4.2×10^3	0.08	9.4
3. " " ‡	10.0	5.0×10^6§	4.5×10^3	0.09	10.2

* See foot-note to Table I.

† The specific activity of added $C^{14}H_3C^{14}OONa = 7.6 \times 10^5$ counts per minute per mg.

‡ For explanation of these control experiments see the text.

§ The specific activity of added $C^{14}H_3C^{14}OONa = 1.6 \times 10^6$ counts per minute per mg.

Incorporation of P^{32} into Phospholipides

Thoracic aortas were excised from female rats 14 to 18 months of age as already described (7). The aortas from eight to twelve rats were blotted, weighed, and transferred to an Erlenmeyer flask containing a bicarbonate-Ringer's solution and inorganic phosphate labeled with P^{32}.

The amounts of the P^{32} incorporated into phospholipides are recorded in Table III. The values in Column 6 are percentages of the added radioactivity recovered as phospholipide per gm. of wet tissue. In the presence of 0.9 gm. of rat thoracic aorta (Experiment 1), approximately 0.7 per cent of the added P^{32} was converted to phospholipide in 2 hours, or 0.8 per cent per gm. of tissue. Under identical conditions, 0.4 gm. of liver slices converted about 2 per cent of the P^{32} to phospholipide, or about 5 per cent per gm. of slices.

The mg. of phospholipide formed from the inorganic phosphorus of the medium were calculated, and the values obtained are recorded in Column 7. Since inorganic phosphorus and various phospholipide intermediates already present in the tissue itself are undoubtedly converted to phospholipide, these values represent but a minimum of the total amount of phospholipide formed by the tissues.

In Experiment 2, 0.02 M succinate was added to the medium containing the aortas. Despite the fact that this substrate increases the respiration of arterial tissue (7), it had no effect on the rate of incorporation of phosphate into phospholipide.

TABLE III

Synthesis of Radioactive Phospholipide by Surviving Rat Thoracic Aorta and Liver Slices

Incubated for 2 hours at 37.5° in Krebs-Henseleit buffer, pH 7.4; 0.18 mg. of P per bath; 3 μc. of P^{32} in each bath.

Experiment No.	Surviving rat tissue			Per cent of added P^{32} recovered as phospholipide		Minimal amounts of newly formed phospholipide per gm. tissue*
	Type	Amount used per bath	Phospholipide content	Total	Per gm. tissue	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
		gm.	*per cent wet weight*			*mg.*
1	Artery	0.90	0.4	0.74	0.82	0.04
	Liver	0.38	2.9	1.75	4.60	0.21
2†	Artery	0.92	0.4	0.70	0.76	0.03
	Liver	0.30	2.8	1.75	5.83	0.26
3	Artery	0.70	0.6	0.59	0.84	0.04
	"	0.55	0.6	0.50	0.91	0.04
	"	0.72	0.5	0.71	0.98	0.04
	Liver	0.51	2.5	2.28	4.46	0.20

* Obtained as follows: 0.82/100 × 0.18 × 25, where 0.82 is the corresponding value in Column 6, 0.18 is the mg. of P per bath, and 25 is the P content of phospholipide.

† 0.02 M succinate added to bath.

DISCUSSION

There can be little doubt that the atheromatosis that develops in animals in which a hypercholesterolemia has been induced experimentally results, in part if not wholly, from an infiltration of circulating lipides into the arterial wall. In the development of the lesion, experimentally induced either by the feeding of cholesterol or by the injection of stilbestrol, it would appear, therefore, that the artery plays a passive rôle in that the lipides composing the atheromatous plaque are not products of arterial metabolism. Since, however, atherosclerosis develops in man in the absence of a raised lipide level of the blood, several investigators have proposed that intrinsic factors must be concerned with the development of this spontaneous lesion (9). The results of the present investigation can leave no doubt that arterial tissue is not inactive in the metabolism of lipides. Its capacity to synthesize fatty acids from the 2-carbon fragment, acetate, and to incorporate phosphate into the phospholipide molecule (a process that involves the formation of two ester bonds) is clearly demonstrated by the results presented here. The significance of our findings with regard to the development of arteriosclerosis in man and animals is difficult to assess, because the rat does not develop arteriosclerosis. These findings do suggest, however, that the proposed concepts in which the artery is considered *passive* may require revision.

EXPERIMENTAL

Preparation of Bicarbonate-Ringer's Solution—The bicarbonate-Ringer's solution was prepared according to the method of Krebs and Henseleit (10). In the fatty acid experiments, each 5 cc. of the buffer contained approximately 1 mg. of sodium acetate, both carbons of which were labeled with C^{14}. In the phospholipide experiment, P^{32} in the form of inorganic phosphate was added to the Ringer's solution. Each flask contained 0.18 mg. of phosphorus in the form of KH_2PO_4 and approximately 3 μc. of P^{32}.

The bicarbonate-Ringer's solutions were saturated with a gas mixture consisting of 95 per cent O_2 and 5 per cent CO_2. The pH of the medium was adjusted to 7.4 just before the addition of the arterial tissue. Immediately after addition of arterial tissue to the flasks, the atmosphere of the flasks was displaced by the same gas mixture. The flasks were incubated in a constant temperature bath at 37.5° for a period of 2 or 3 hours.

Extraction of Radioactive Fatty Acids and Cholesterol—The methods employed for the isolation of these lipides and the determination of their radioactivity have been described elsewhere (11).

Extraction of Phospholipide—5 cc. of ice-cold 0.3 N trichloroacetic acid were added to each flask to stop the reaction. The contents of the flask were then transferred to an all-glass homogenizer, together with 1 or 2 cc. of the cold trichloroacetic acid, and the tissue thoroughly macerated. The tube and its contents were centrifuged and the supernatant discarded. The grinding and centrifugation of the precipitate were repeated twice. The acid-washed precipitate was then extracted five times with a 3:1 alcohol-ether mixture at 50° and twice with ethyl ether. All extracts were combined in a 250 cc. Erlenmeyer flask that had a small side arm. The extracts were concentrated to a small volume on a steam bath. About 5 cc. of distilled water and 50 mg. of KH_2PO_4 were then added to the flask. This was done in order to dilute any inorganic P^{32} which may have been carried into the extracts. The concentration was continued until alcohol was no longer detected. Ethyl ether was added to the flask and the flask vigorously shaken. The supernatant ether layer was poured off, the lower water phase being caught in the side arm. The latter was then reextracted twice and all three ether extracts combined in an Erlenmeyer flask which also had a side arm. In order to remove any remaining inorganic P^{32}, the volume of the combined extracts was reduced to about 15 cc., on a hot water bath, and 50 mg. of KH_2PO_4 again added with 5 cc. of water. After the flask was shaken violently, the ether phase was separated from the water phase by means of the side arm and the water phase was extracted twice with ethyl ether. The extracts were combined in another side-armed flask. The water phase was tested for radioactivity, which was found to be negligible. The volume of the ethyl ether extracts was again reduced to 15 to 20 cc. and 5 cc. of water were added to wash the ethyl ether extracts free of inorganic phosphate. The ethyl ether was transferred to a 100 cc. volumetric flask and made to volume. Aliquots of the ether solution were mounted on blotting paper for determination of radioactivity (12). The P^{31} content of other aliquots was measured by King's method (13), the color being determined by the Klett-Summerson colorimeter.

SUMMARY

1. The synthesis of lipides by the rat artery was investigated with the aid of P^{32} and acetic acid in which both carbons were labeled with C^{14}.

2. Surviving rat thoracic aorta is capable of converting acetate to fatty acids. The incorporation of P^{32} into the phospholipide molecule is also readily accomplished by the rat artery.

3. The bearing of these synthetic reactions on the development of atherosclerosis is discussed.

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THE ADENOSINETRIPHOSPHATASE AND PHOSPHATASE (ACID AND ALKALINE) ACTIVITY OF MUSCLE HOMOGENATES FROM RABBITS ON A VITAMIN E-DEFICIENT DIET

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The repeated observation of an increased oxygen consumption by dystrophic muscle from vitamin E-deficient animals (1–6) has stimulated a search for the enzyme system or systems which might be responsible. Houchin (7) reported an increased succinoxidase activity of hamster muscle and an inhibition of this activity by α-tocopheryl phosphate. Though the inhibitory effect of α-tocopheryl phosphate on the *in vitro* succinoxidase activity of dystrophic hamster muscle, as well as normal muscle, was observed by subsequent workers (8), no significant difference in the activity of this enzyme, however, was found in the dystrophic muscle as compared to normal muscle. From the work of Govier *et al.* (9, 10) it appears that α-tocopheryl phosphate might also inhibit coenzyme I nucleotidase and lactic dehydrogenase. Spaulding and Graham (11) have likewise found that α-tocopheryl phosphate inhibits the coenzyme I nucleotidase.

A decreased creatine content in dystrophic muscle, resulting from a vitamin E-deficiency, has also been well substantiated (12–15). Creatine is regarded as existing in muscle as phosphocreatine and only transitorily and to a relatively small extent as creatine. If this is true, then it follows that it is the phosphocreatine which is decreased. Assuming for the moment that there is no decreased synthesis of creatine in the vitamin E-deficient animal, at least three possible explanations present themselves for the decreased phosphocreatine concentration in the dystrophic muscle: (1) the creatine does not enter the muscle cells rapidly enough, (2) there is an accelerated breakdown of phosphocreatine within the muscle, and (3) the rate of phosphocreatine regeneration is decreased. The recent report by Hummel (16) indicates that the oxidative phosphorylation of creatine in muscle may be decreased in nutritional muscular dystrophy. In the same paper Hummel reports a decreased adenosinetriphosphatase activity of muscle homogenates from dystrophic hamsters and guinea pigs. He, however, foresaw a possible explanation for his observations: "... it must be emphasized that there is a smaller active mass of muscle as a result of necrosis and fibrotic infiltration which may, in part, explain the lowered enzymatic activity in advanced dystrophy."

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In the course of studies on nutritional muscular dystrophy in rabbits in this laboratory, the adenosinetriphosphatase and phosphatase (acid and alkaline) activity of muscle homogenates was determined. It is generally regarded that adenosine triphosphate is resynthesized by means of the Lohmann reaction, in which phosphocreatine supplies the energy-rich phosphate bond, as well as by oxidative phosphorylation. The thought occurred to us that perhaps in the dystrophic animal, in the initial stages of the disorder, adenosine triphosphate was more rapidly broken down than in the normal animal. The call on phosphocreatine might therefore be greater to resynthesize adenosine triphosphate. If the rate of adenosine triphosphate breakdown were such that even the increased rate of oxygen consumption by the muscle was inadequate to generate sufficient energy for resynthesis of the required energy-rich phosphate bonds, there could be an initial increase in the concentration of free creatine. If then the muscle does not hold creatine as such for any length of time, or holds only a limited amount of free creatine, one would have an explanation for the eventual decrease in creatine (phosphocreatine) concentration of the muscle. The leakage of creatine from the muscle might also be responsible for the creatinuria in animals with nutritional muscular dystrophy, if one keeps in mind the demonstration by Borsook and Dubnoff (17) that creatine is converted *in vitro* to creatinine at a relatively slow rate.

Since phosphate esters are intimately associated with carbohydrat metabolism and probably with metabolism in general, it seemed logical t investigate the relatively non-specific phosphatases too.

EXPERIMENTAL

Male rabbits weighing from 572 to 1390 gm. were placed on the dystrophy-producing diet of Goettsch and Pappenheimer (18). Some of these rabbits, the controls, received orally 10 mg. of α-tocopherol[1] in ethyl laurate per day. The α-tocopherol was administered at least 3 hours after removal of uneaten food in the morning and at least 3 hours before a fresh portion of the diet was given in the evening (19). The animals were weighed every other day.

For the quantitative collection of urine specimens, the bladder of the animal was emptied by gentle pressure on the abdominal wall; the animal was then placed in a metabolism cage for 6 hours. During this period of time only water was available. The urine excreted during this period combined with that obtained by gentle pressure on the abdominal wall at the end of the period was regarded as a 6 hour specimen.

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[1] Some of the α-tocopherol was kindly supplied by Dr. Dana S. Crum, Merck and Company, Inc., Rahway, New Jersey. The remainder was purchased from General Biochemicals, Inc., Chagrin Falls, Ohio,

When possible, creatinine and total creatinine were determined immediately; otherwise the samples were stored at 0° and analyzed within 24 hours. The creatinine and total creatinine were determined by adaptation of the method of Folin (20) to the Klett-Summerson photoelectric colorimeter.

The animals were continued on the diet until there was a definite increase in the creatine excretion for 3 or more days. They were then sacrificed by a blow at the back of the head. As quickly as possible a piece of muscle from the right thigh was snipped out; approximately 500 mg. were weighed and homogenized with cold doubly distilled water in a small all-glass tissue mincer. After further dilution, the homogenate was used in the determination of adenosinetriphosphatase activity within 15 minutes of the death of the animals. The same homogenate was stored at 0° and used for the phosphatase determinations within 2 hours after the sacrifice of the animal.

The adenosinetriphosphatase activity of the muscle homogenates was determined according to the method of DuBois and Potter (21) except that the volumes of the reagents were increased 5 times. Adenosine triphosphate, which served as a substrate in this determination, was prepared according to the directions of LePage (22). It had on analysis an inorganic phosphate content of 0.26 per cent. Of the total phosphorus (corrected for inorganic phosphate-phosphorus initially present) 65 per cent was released in 7 minutes by hydrolysis at 100° in N hydrochloric acid. Under the same conditions, 76.5 per cent was released in 10 minutes. In the case of four control and four experimental animals, 1 mg. of disodium *dl*-α-tocopheryl phosphate[2] in 0.5 ml. of distilled water replaced an equal volume of water in the reaction mixture. When the tocopheryl phosphate was thus used, the reaction mixture was filtered instead of centrifuged prior to the determination of inorganic phosphate; the suspended material could not be removed satisfactorily by centrifugation.

Acid, pH 4.50, and alkaline, pH 9.78, phosphatase activities of the muscle homogenates were determined with the method proposed by Binkley *et al.* (23) for serum phosphatases, except, of course, that a muscle homogenate was used instead of serum. The pH of the buffers was determined with a glass electrode. The substrate was disodium phenyl phosphate.

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[2] The disodium *dl*-α-tocopheryl phosphate was kindly given to this laboratory by Dr. Kenneth L. Zierler and Dr. J. L. Lilienthal, Jr., of The Johns Hopkins University Hospital, Baltimore, Maryland.

All determinations of activity were run in quadruplicate. In the instances in which tocopheryl phosphate was used, 10 mg. of the disodium salt were dissolved in 5 ml. of doubly distilled water and replaced this volume of the stock buffer solution in the preparation of 100 ml. of the substrate-buffer solution.

TABLE I

Effect of Vitamin E-Deficient Diet on Weight and Creatinine and Creatine Excretion of Young Rabbits

Rabbit No.	Weight			Creatinine			Creatine			Days on diet	Condition of animal when sacrificed
	Initial	Final	Maximal	Initial	Final	Maximal	Initial	Final	Maximal		
					Vitamin E-deficient						
	gm.	*gm.*	*gm.*	*mg. per 6 hrs.*	*mg. per 6 hrs.*	*mg. per 6 hrs.*	*mg. per 6 hrs.*	*mg. per 6 hrs.*	*mg. per 6 hrs.*		
2	830	1160	1340	8.3	4.5	12.8	2.9	12.0	12.2	27	Dead 60 min. when sampled
3	1060	1510	1675	12.7	12.2	17.2	0.3	24.0	25.8	34	No signs of weakness
5	1360	1640	1730	14.2	12.5	18.5	5.0	20.5	26.2	33	Hind quarters weak
6	780	910	1320	9.0	9.0	12.0	1.5	34.0	34.0	25	Apparently well
7	750	940	1310	9.0	3.7	11.2	2.5	23.8	23.8	29	Moribund
11	662	1020	1180	6.8	9.2	10.0	1.0	20.6	21.7	29	Weak but alert
13	572	1095	1125	5.2	6.2	12.5	2.8	18.3	22.5	31	Slight signs of weakness
14	785	1010	1080	8.5	9.7	11.2	0.7	19.8	19.8	19	Weak
18	1230	1400	1400	11.2	8.7	11.2	1.8	14.5	24.3	23	“ but alert
19	1025	1260	1285	10.2	16.2	16.2	4.2	13.8	13.8	36	Apparently well
22	1020	1255	1255	9.2	14.0	14.0	1.2	7.8	16.8	33	“ “
					Control[*]						
4	1390	1570	1570	14.2	11.3	14.2	5.0	5.0	5.0	36	In good health
9	785	1210	1220	8.3	14.0	14.0	3.4	4.7	4.7	28	“ “ “
10	785	960	960	4.8	8.2	8.2	1.7	2.5	2.5	15	“ “ “
15	1280	1500	1500	14.7	14.2	16.0	2.3	2.5	2.5	20	“ “ “
16	1240	1650	1650	13.2	15.0	15.0	3.0	0.7	3.0	22	“ “ “
17	1380	1480	1480	14.5	12.5	18.0	0.2	3.0	4.5	21	“ “ “
20	1065	1220	1220	9.0	12.2	12.2	1.2	0.4	4.5	29	“ “ “

[*] The control rabbits received orally 10 mg. per day of α-tocopherol in ethyl laurate.

RESULTS AND DISCUSSION

Summarized in Table I are the data on weight and creatinine and total creatinine excretion of the rabbits used in this study.

The control and dystrophic rabbits appear to show an approximately similar adenosinetriphosphatase activity (Table II). An exception is seen in the case of Rabbit 2, whose adenosinetriphosphatase activity was found to be relatively low. This may be due to the fact that this animal had died within the hour previous to taking the muscle sample and the time, therefore, which elapsed between assay and death was different from that in all the other instances. It may also, however, be an indication that in

TABLE II

Adenosinetriphosphatase Activity of Muscle Homogenates from Rabbits on Vitamin E-Deficient Diet

The activity is expressed as micrograms of P per mg. of wet muscle per 15 minutes.

Rabbit No.	Without α-tocopheryl phosphate		With α-tocopheryl phosphate	
	With calcium	Without calcium	With calcium	Without calcium
Control*				
4	15.14	4.94		
9	15.10	7.75		
10	16.12	7.60		
15	16.16	6.34	12.72	
16	11.91	5.24	11.64	1.93
17	15.36	6.19	14.97	4.27
20	14.20	7.91	13.52	4.35
Average	14.85 ± 0.38	6.58 ± 0.31	13.21 ± 0.47	3.52 ± 0.54
Range	11.91–16.16	4.94–7.91	11.64–14.97	1.93–4.35
Experimental				
2	7.45	3.70		
3	18.73	5.45		
5	16.65	6.43		
6	16.05	8.75		
7	12.75	6.75		
11	11.10	4.60		
13	14.31	5.21		
14	15.17	7.14	12.43	4.07
18	11.36	4.87	8.67	1.99
19	15.96	4.80	14.28	3.13
22	18.67	7.02	13.82	3.75
Average	14.35 ± 0.70	5.88 ± 0.30	12.30 ± 0.85	3.23 ± 0.31
Range	7.45–18.73	3.70–8.75	8.67–14.28	1.99–4.07

* The control rabbits received orally 10 mg. of α-tocopherol in ethyl laurate per day.

the rabbit, as in the guinea pig and hamster, adenosinetriphosphatase activity is decreased in a severely dystrophic state (16). The rabbit was definitely in the terminal stages of dystrophy, as evidenced by physical signs. The value for Rabbit 2 is included in the average value; were it to be excluded, the average value would still not be markedly different, namely 15.07 γ.

With the thought in mind that α-tocopherol has been shown to participate in biological systems and not merely as a non-biological oxidant (5, 7–10), disodium *dl*-α-tocopheryl phosphate was added to the substrate-buffer mixture in the case of four control and four dystrophic rabbits. As can be seen from Table II, in the instance in which added calcium and the tocopheryl phosphate are both present, the results are at most only suggestive that the enzyme, adenosinetriphosphatase, may be less active in the presence of the tocopheryl phosphate. When calcium was not added to the reaction mixture, the activity of the enzyme was definitely lower in the presence of the tocopheryl phosphate. Since calcium α-tocopheryl phosphate is relatively insoluble, a test was made of the possibility that the observed decreased activity of adenosinetriphosphatase was due to a removal of the calcium originally present in the homogenate. The results of this test are given in Fig. 1.

One might interpret the findings presented in Fig. 1 as strongly indicative that α-tocopheryl phosphate exerts its inhibitory effect on adenosinetriphosphatase because of its precipitation of the calcium, which is an essential cofactor for the enzyme.

Observations on the acid and alkaline phosphatase activities of muscle homogenates from normal and dystrophic rabbits are summarized in Table III. In the case of the alkaline phosphatase, the range and the overlapping of the activity values, found in the rather limited series of animals used, do not, at present, allow for any conclusion. There does, however, appear to be a definite increase in the acid phosphatase activity of the muscle homogenates from the dystrophic rabbits. It is worthy of note that only in the case of one dystrophic animal, Rabbit 11, is the acid phosphatase activity as low as the highest value found in the control series.

The data in Table III, in regard to the effect of α-tocopheryl phosphate on the acid phosphatase activity of muscle homogenates, indicate that, under the conditions of the determination, less phenyl phosphate, the substrate, is hydrolyzed when α-tocopheryl phosphate is present than when the latter is absent. That α-tocopheryl phosphate might interfere in the determination of the phenol released from phenyl phosphate suggested itself as a possible answer to the observed decreased activity. To settle this point the experiment presented in Table IV was set up. It is clear from the data in Table IV that α-tocopheryl phosphate does not interfere with the development of the blue color given by phenol (or tyrosine) with the reagent of Folin and Ciocalteau. In this experiment the tubes containing the α-tocopheryl phosphate (1 mg. of the disodium salt in each) were turbid, owing to the precipitation of this material. The precipitate, how-

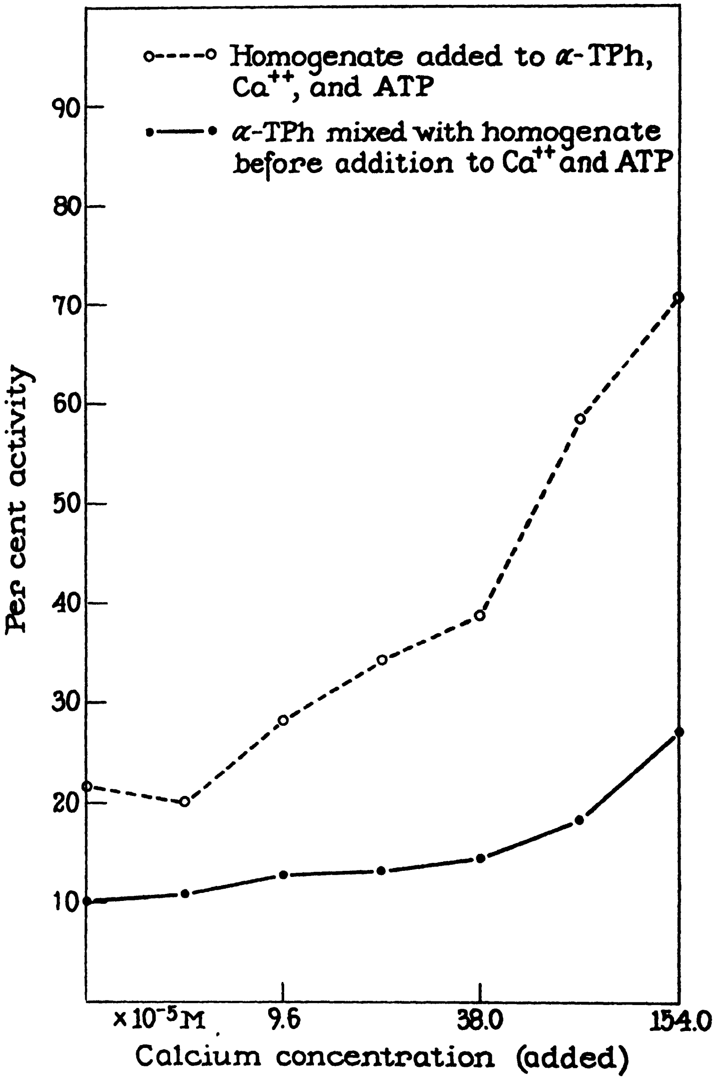

FIG. 1. The effect of calcium on the inhibition by α-tocopheryl phosphate (α-TPh) of the adenosinetriphosphatase activity of rabbit muscle homogenates. The calcium was added as calcium chloride. The concentration of α-tocopheryl phosphate was 3.4×10^{-4} M in each instance.

TABLE III

Phosphatase Activity of Muscle Homogenates from Rabbits on Vitamin E-Deficient Diet

The activity is expressed as micrograms of phenol liberated per hour by 10 mg. of muscle (wet weight) at 37°.

Rabbit No.	Acid phosphatase		Alkaline phosphatase
	Without α-tocopheryl phosphate	With α-tocopheryl phosphate	
Control[*]			
4	2.60		1.20
9	3.45		2.83
10	2.13		2.00
15	1.97	0.00[†]	
16	2.15	0.60	
17	1.73	0.73	
20	2.77	1.35	
Average	2.40 ± 0.15	0.67 ± 0.19	2.01 ± 0.32
Range	1.73-3.45	0.00-1.35	1.20-2.83
Experimental			
2	3.94		2.32
3	4.41		1.50
5	4.11		0.25
6	3.70		3.57
7	4.45		3.01
11	3.40		1.80
13	4.47		0.80
14	4.17	0.00[†]	
18	4.10	2.37	
19	7.97	1.17	
22	5.75	2.20	
Average	4.58 ± 0.26	1.43 ± 0.38	1.89 ± 0.30
Range	3.40-7.97	0.00-2.37	0.25-3.57

[*] The control rabbits received orally 10 mg. of α-tocopherol in ethyl laurate per day.

[†] 1 mg. of disodium *dl*-α-tocopheryl phosphate was present in each tube; in all of the other determinations only 0.5 mg. of the disodium *dl*-α-tocopheryl phosphate was used per tube.

ever, was finely dispersed, as evidenced by the relatively constant increment in the density reading of each tube. This turbidity was never observed when muscle homogenates were part of the mixture and filtration was employed.

TABLE IV

Effect of Added Disodium dl-α-Tocopheryl Phosphate on Color Developed with Known Amounts of Phenol and Phenol Reagent of Folin and Ciocalteau

Each tube in Column 3 contained 1 mg. of the disodium *dl*-α-tocopheryl phosphate (TPh) in addition to the indicated concentration of phenol.

Phenol concentration (1)	Photometer reading (2)	Photometer reading in presence of 1 mg. TPh* (3)	Photometer reading in presence of 1 mg. TPh† (4)
mg.			
0.000	0.000	0.119	0.000
0.000	0.000	0.119	0.002
0.004	0.063	0.184	0.065
0.004	0.062	0.182	0.065
0.008	0.121	0.243	0.128
0.008	0.122	0.240	0.126
0.012	0.180	0.310	0.198
0.012	0.182	0.300	0.187
0.016	0.240	0.365	0.249
0.016	0.242	0.358	0.241
0.020	0.299	0.420	0.300
0.020	0.301	0.421	0.302

* These readings were taken with the photometer set at zero with the tubes of Column 2 containing no phenol.

† These readings were taken with the photometer set at zero with the tubes of Column 3 containing no phenol.

TABLE V

Rôle of Disodium dl-α-Tocopheryl Phosphate as Substrate for Rabbit Muscle Acid Phosphatase

A 1 per cent muscle homogenate served as the source of the enzyme. The concentration of disodium *dl*-α-tocopheryl phosphate in the presence of the enzyme was 10^{-3} M; of disodium phenyl phosphate, 10^{-3} M. An acetate buffer, pH 5.0, was used. Incubation was at 37° for 1 hour. The activities are expressed in terms of 10 mg. of wet muscle. The muscle samples had been in a deep freeze box for 1 to 2 weeks before use. The figures in parentheses are calculated values for phosphorus, in micrograms, from the corresponding observed values for phenol.

Experiment No.	α-Tocopheryl phosphate	α-Tocopheryl phosphate and disodium phenyl phosphate	Disodium phenyl phosphate	
	γ P per hr.	*γ P per hr.*	*γ P per hr.*	*γ phenol per hr.*
1	1.50	0.80	4.70	9.30 (3.10)
2	0.14	0.00	4.47	11.45 (3.80)
3	0.00	0.00	4.67	13.95 (4.60)
4	0.00	0.98	4.48	12.60 (4.16)

The further possibility that α-tocopheryl phosphate might act as a substrate for the acid phosphatase of the muscle, substituting in part for the phenyl phosphate, came to mind. A determination of the phenol released by acid phosphatase from phenyl phosphate in the presence of α-tocopheryl phosphate might indicate only the fractional activity of the enzyme. If

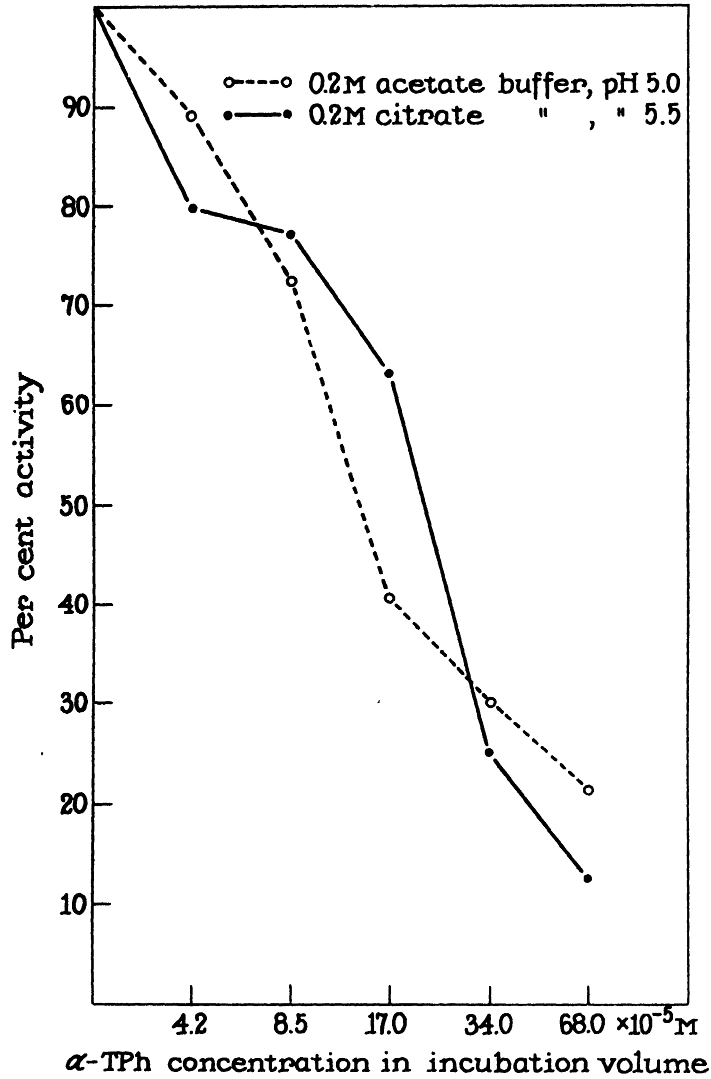

FIG. 2. The effect of α-tocopheryl phosphate (α-TPh) concentration on the acid phosphatase activity of rabbit muscle homogenate. The α-tocopheryl phosphate was added as a solution of its disodium salt.

the enzyme affected the hydrolysis of α-tocopheryl phosphate, as well as phenyl phosphate, a determination of the phenol alone would not take this into account. To settle this point the experiments summarized in Table V were carried out. Here, not only the phenol released from phenyl phos-

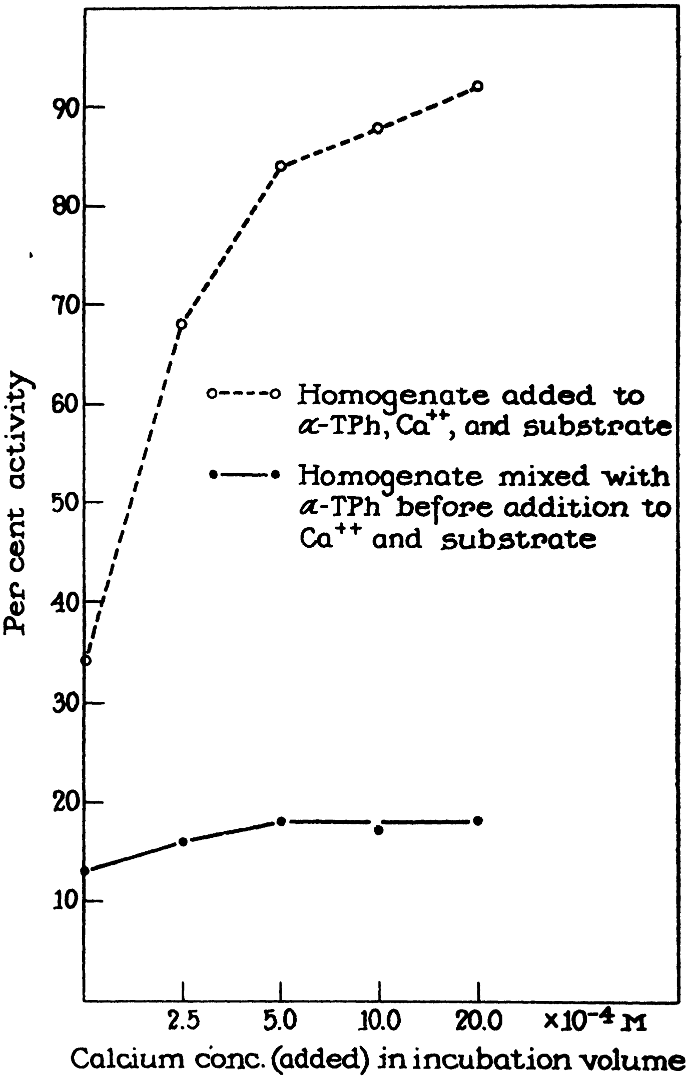

FIG. 3. The effect of calcium on the inhibition by α-tocopheryl phosphate (α-TPh) of the acid phosphatase activity of rabbit muscle homogenates. The calcium was added as calcium chloride; the α-tocopheryl phosphate, as the disodium salt. The concentration of α-tocopheryl phosphate was 2.2×10^{-4} M in each instance.

phate but the increase in inorganic phosphate was determined. It appears from this experiment that the amount of inorganic phosphate liberated when α-tocopheryl phosphate and phenyl phosphate are both present is either very small or negligible. The α-tocopheryl phosphate is not used to any extent as a substrate by the muscle acid phosphatase. It may very well be that α-tocopheryl phosphate combines with the enzyme or some essential component which is necessary for the activity of the acid phosphatase and thereby inhibits the activity of the enzyme on the substrate phenyl phosphate, with which the enzyme is likewise confronted.

The inhibitory effect of α-tocopheryl phosphate on the acid phosphatase activity of rabbit muscle homogenates was found to vary with the concentration of α-tocopheryl phosphate, as shown in Fig. 2.

Calcium, when added as the chloride so that the final concentration of this cation ranged from 2.5×10^{-4} M to 4.0×10^{-3} M, was found to have no apparent effect on the activity of the rabbit muscle acid phosphatase. Calcium, however, as shown in Fig. 3, could decrease the inhibitory effect of α-tocopheryl phosphate. The inhibition by α-tocopheryl phosphate of the acid phosphatase activity decreased as the calcium concentration increased, provided that the α-tocopheryl phosphate was added to the calcium-containing buffer-substrate solution (upper curve, Fig. 3). If, on the other hand, the α-tocopheryl phosphate was added to the muscle homogenate before the latter was added to the calcium-containing buffer-substrate solution, only a slight effect of the calcium was demonstrable (lower curve of Fig. 3). It would seem that the α-tocopheryl phosphate when added to the muscle homogenate combined with some component in the homogenate, either the acid phosphatase or some unknown cofactor of this enzyme, and thereby affected the activity. The affinity of α-tocopheryl phosphate for this undetermined component appears to be a relatively strong one: calcium, even in excess, did not result in the precipitation of the rather insoluble calcium tocopheryl phosphate.

SUMMARY

No definite increase or decrease was observed in the adenosinetriphosphatase and alkaline phosphatase activities of muscle homogenates from dystrophic rabbits.

A definite increase, an approximately 2-fold increase, in the acid phosphatase activity of muscle homogenates from dystrophic rabbits was observed.

The *in vitro* addition of α-tocopheryl phosphate to the adenosinetriphosphatase system resulted in a decrease of activity. The inhibitory effect of α-tocopheryl phosphate appears to be due to the precipitation of calcium, which serves as an activator (cofactor) for this enzyme.

The addition of α-tocopheryl phosphate *in vitro* to the muscle acid phosphatase system resulted in an inhibition of activity. Calcium is not required as a cofactor for the maximal activity of the muscle acid phosphatase under the conditions employed. Though it was found possible to decrease the inhibitory effect of α-tocopheryl phosphate on the enzyme by the addition of calcium to the system, it was also possible to demonstrate that the inhibition by α-tocopheryl phosphate of the muscle acid phosphatase was but slightly affected if the α-tocopheryl phosphate was mixed with the muscle homogenate before calcium was added.

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THE EFFECT OF ACETIC ACID ON THE STABILITY OF SERUM PROTEINS*

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(Received for publication, December 27, 1948)

Several investigators (1, 2) have shown that when horse serum is denatured a change in the relative distribution of electrophoretic components occurs. Van der Scheer, Wyckoff, and Clarke (1) found that heating of serum at 65° resulted in the formation of a single component, with the disappearance of the albumin and globulin peaks of the serum. This was confirmed by Davis, Hollaender, and Greenstein (2) who also demonstrated a similar phenomenon produced by ultraviolet radiation.

In the present study the stability of serum and certain plasma protein fractions at acid pH values in the presence of acetate has been investigated. To determine the stability of these proteins, electrophoretic analysis was carried out after removal of the acetate ions and the results are compared with those obtained with undenatured human serum.

EXPERIMENTAL

Usually three or four different samples of normal human serum were pooled and adjusted to the desired pH by dropwise addition of acetic acid with sufficient stirring to minimize any possible effects of local excess of acid. After standing at room temperature for 30 minutes, the solutions were dialyzed at 3–5° against 2000 cc. of 0.15 M sodium chloride for 2 days with several changes of the dialysate. The samples were then diluted with 0.1 N sodium diethyl barbiturate buffer of pH 8.6 to a final protein concentration of 1.87 per cent, *i.e.* 3.0 mg. of protein nitrogen per cc., and dialyzed in the cold for 3 days against liberal portions of this buffer.

The electrophoretic experiments were performed at 1.5° in a Tiselius apparatus (3) equipped with the schlieren scanning device of Longsworth (4). The concentrations of the electrophoretically separable components were estimated by the procedure of Tiselius and Kabat (5). The results are expressed as the ratio of the area of each component to the total area, exclusive of the δ- and ε-boundaries. Average values of the descending and ascending boundaries are given. Mobilities were computed from the descending patterns and refer to 0°.

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Results

In Fig. 1 are superimposed the tracings of the electrophoretic patterns of normal human serum before and after exposure to acetic acid at pH 3.0 for 30 minutes. Electrophoresis of the acetic acid-treated serum showed a progressive increase in homogeneity of the material with the formation of a new peak. This new component, designated as D (for denatured), migrates with a mobility intermediate to mobilities of the α_2- and β-globulins.

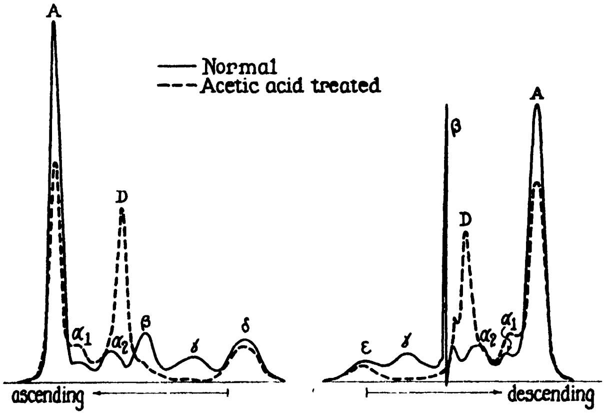

FIG. 1. Superimposed tracings of a 1.87 per cent solution of normal human serum before and after treatment with acetic acid at pH 3.0. Electrophoresis was carried out in a sodium diethyl barbiturate buffer at pH 8.6 and 0.1 ionic strength at a potential gradient of 5.3 volts per cm. for 12,600 seconds.

Exposure to acetic acid at pH 3.0 for 30 minutes was found to be necessary for the appearance of this component. In samples treated for 30 minutes, the D component has the same mobility as that of a sample treated for 48 hours, although in the latter case the D component is present in a somewhat higher concentration.

Effect of pH—The striking differences of the two patterns of Fig. 1 made it desirable to test the effects of acetic acid at various pH values. Table I shows the apparent distribution of the protein components observed in six experiments in which the pH was varied between 4.0 and 2.8. After exposure to pH 4.0, no significant changes were found. At pH 3.7, the α_1-globulin increased at the expense of the albumin. pH 3.4 causes a further shift in the concentration of these two components, and the D component appears. At pH 3.1, the amount of γ-globulin is reduced. These results indicate clearly that in the case of normal human serum in an acetic acid medium pH 4.0 represents the critical pH for the stability of the proteins. At pH values below 4.0 denaturation occurs.

In a series of experiments in which hydrochloric acid, lactic acid, or equilibration with a glycine-hydrochloric acid buffer was used for adjustment to pH 3.0, the formation of the D component was not observed. In these cases all the electrophoretic components of normal serum were present. However, a slight shift in the distribution of the relative concentrations of components was noticeable which was most marked in the case of the lactic acid-containing medium.

Stability of Plasma Fractions—Since it was found that the serum globulins are more specifically affected by the presence of acetic acid, the stability of plasma fractions was investigated. Various preparations were tested.[1] Only in Fraction IV-1, which contains α_1-, α_2-, and β-globulins, traces of albumin and γ-globulin, and which is characterized by a lipide content of 15.0 per cent, was a change similar to the one in serum noticed. This suggests the lability of lipide-protein linkages as the cause of the D peak. Another observation seems worth recording. In Fig. 2 are shown the electrophoretic patterns of γ-globulin before and after treatment with acetic acid at pH 3.0 for 30 minutes. The average mobility, $u = -1.2 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}, remains unchanged. The somewhat greater homo-

TABLE I

Electrophoretic Distribution of Proteins in 1.87 Per Cent Solution of Normal Human Serum after Exposure to Acetic Acid at Various pH Values for 30 Minutes

Pretreatment of serum		Concentration in per cent as						Mobilities × 10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$					
Concention of acetic acid	pH	Albumin	Globulins					Albumin	Globulins				
			α_1	α_2	D	β	γ		α_1	α_2	D	β	γ
N													
		58.3	5.2	10.4		14.0	12.1	−6.4	−5.2	−4.1		−3.0	−1.3
0.294	4.06	57.1	5.6	12.1		13.2	12.0	−6.4	−5.2	−4.1		−3.0	−1.2
0.735	3.70	53.1	9.4	14.8		11.0	11.7	−6.3	−5.1	−4.0		−3.1	−1.2
1.47	3.40	47.0	14.9		28.0		10.1	−6.3	−5.2		−3.7		−1.2
2.94	3.10	45.0	16.0		34.8		4.2	−6.3	−5.3		−3.6		−1.2
5.88	2.80	47.8	10.0		40.8		1.4	−6.3	−5.2		−3.6		−1.3

Electrophoresis was carried out in a sodium diethyl barbiturate buffer at pH 8.6 and 0.1 ionic strength.

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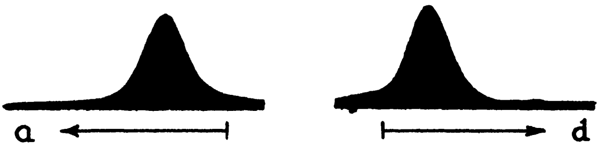
[1] The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard Medical School.

geneity of the pattern in Fig. 2, *B*, however, is accompanied by an increase of irreversibly denatured protein which, in the presence of neutral salt, precipitates in the neighborhood of the isoelectric pH of this protein. Moreover, it seems of interest to consider this result in connection with the one described above; namely, that exposure of purified γ-globulin to pH 3.0 in the presence of acetic acid does not alter the mean mobility of the protein fraction, whereas the same treatment causes this component to disappear in the serum.

A. Normal



B. Acetic acid treated

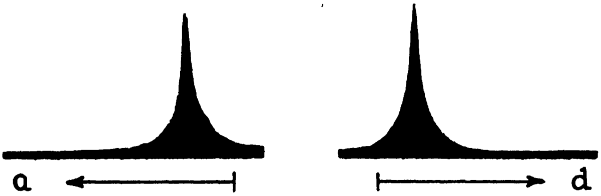


FIG. 2. Electrophoretic patterns of human γ-globulin. The patterns were obtained from a 1.0 per cent protein solution in sodium diethyl barbiturate buffer at pH 8.6 and 0.1 ionic strength at a potential gradient of 5.2 volts per cm. for 10,800 seconds.

DISCUSSION

It is apparent from the results presented in this paper that marked alterations occur in the distribution of electrophoretic components of serum after exposure to acetic acid for 30 minutes. These changes occur in a relatively short time and are dependent on the pH of the solution. No experiments to give a detailed account of the nature of this particular denaturation have been carried out. From the fact that exposure to acetic acid of the purified plasma fractions, *e.g.* albumin and γ-globulin, does not produce a significant shift of the electrophoretic mobilities, we may assume that the prevailing experimental conditions do not alter markedly the number of ionizable groups of these proteins. In the case of the lipide-rich protein fraction, Fraction IV, however, a change in the electrophoretic behavior was noticed. This leads us to believe that fission of lipide-protein linkages may be the cause of this phenomenon. The splitting of such bonds and a simultaneous recombination of molecules may readily yield aggregates of different size, but with a similar charge distribution such a mechanism could easily explain the formation of the D peak observed in the case of serum. A similar result was obtained by Tiselius and Horsfall in their work on the dissociation and reassociation of different hemocyanins (6).

From a comparison of the effect of acetic acid on serum proteins with that of other acids, *i.e.* hydrochloric acid, lactic acid, and the glycine-hydrochloric acid mixture, it is apparent that the formation of the D component at pH 3.0 is relatively specific for the presence of acetate ions. It therefore seems worth while to stress that denaturation does not depend on the nature of the protein and the acidity of the medium alone, but that the type of ions present in the medium also plays a rôle in determining the degree of stability of a protein. Moreover, it was noticed that under any given experimental condition the rate of denaturation may be enhanced considerably by the presence of neutral salts.

SUMMARY

Exposure of serum to acetic acid at pH values below 4.0 causes an irreversible denaturation of the proteins. This phenomenon is dependent on the pH of the solution. At pH 3.0, a new electrophoretic component, designated as D, was observed, which migrates with a mobility intermediate to mobilities of the α_2- and β-globulins.

The authors are indebted to Dr. J. L. Oncley of the Department of Physical Chemistry, Harvard Medical School, Boston, for supplying the plasma fractions.

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THE SYNTHESIS OF DL-TRYPTOPHAN-β-C^{14}, INDOLE-3-ACETIC ACID-α-C^{14}, AND DL-TRYPTOPHAN-3-C^{14} *

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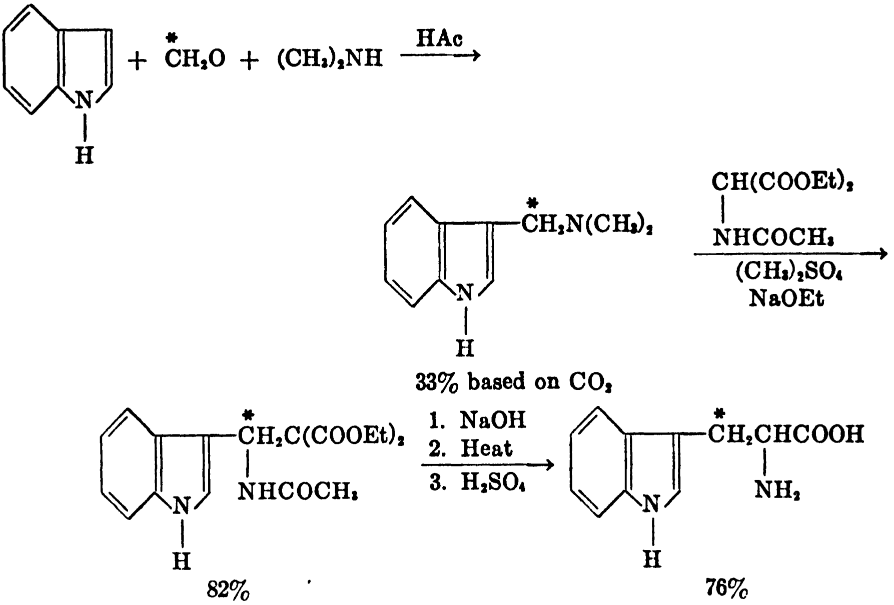
The first recorded synthesis of tryptophan, by Ellinger and Flamand in 1908 (1), involved the condensation of indole-3-aldehyde and hippuric acid in a typical azlactone reaction. The yield in the synthesis was much improved in 1935 by Boyd and Robson (2), who employed hydantoin instead of hippuric acid for the condensation. This reaction has recently been used by Bond (3) in the preparation of DL-tryptophan-carboxyl-C^{14}. In 1944 Snyder and Smith (4) and Albertson, Archer, and Suter (5) independently reported almost identical synthesis of tryptophan by the con-



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* This paper is based on work performed under contract No. W-7405-eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley.

This system of nomenclature is in accord with that proposed by Calvin, Heidelberger, Reid, Tolbert, and Yankwich (13). The β-carbon is in the side chain and the 3-carbon is in the indole ring.

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densation of gramine with ethyl acetamidomalonate, followed by hydrolysis. Suitable modifications of these procedures were employed in the synthesis of DL-tryptophan-β-C^{14}. The isotopic indole acetic acid was prepared from gramine by modifications of a known method (6), which involves the alkylation of potassium cyanide by gramine, followed by hydrolysis. The reactions and the yields obtained are indicated by the equations shown in the accompanying scheme.

The isotopic formaldehyde was prepared from methanol, which was obtained from carbon dioxide by high pressure hydrogenation (7). The preparation of DL-tryptophan-3-C^{14} is indicated in the experimental section.

We are deeply grateful to Mrs. F. Christensen and Dr. B. M. Tolbert for the preparation of the formaldehyde, and to Dr. W. G. Dauben for the benzoic acid-carboxyl-C^{14} used in these syntheses.

EXPERIMENTAL

Copper hydroxide, freshly precipitated from a cupric nitrate[1] solution by the addition of dilute ammonium hydroxide, was washed thoroughly with water and applied to a copper screen, which was rolled, inserted into a small quartz tube, and reduced with hydrogen at 450°. The labeled methanol, which contained water, was passed over the catalyst (600°) with a stream of air from a small bubbler heated to 70°. The reduction products were trapped in a small bubbler containing 1 ml. of water and a trace of methanol. The unabsorbed gases were passed through a combustion furnace to recover the radioactive carbon. The yields of formaldehyde varied from 40 to 60 per cent. The formaldehyde, thus obtained from methanol following the reduction of 25 mM of labeled carbon dioxide, was added to a chilled mixture of 1.42 gm. of 33 per cent aqueous dimethylamine and 1.42 gm. of glacial acetic acid, and the solution was quickly added to 1.10 gm. of indole. Heat was evolved, the indole dissolved, and the mixture was allowed to stand at room temperature for 18 hours. The light yellow solution was then added dropwise to an ice-cold solution of 1.42 gm. of sodium hydroxide in 20 ml. of water, and a white crystalline precipitate of gramine was formed, which melted at 121–126°. Yield, 1.39 gm. (33 per cent based on carbon dioxide); specific activity, 152,000 counts per minute per mg.

The crude gramine, 1.29 gm., was allowed to react at room temperature with a mixture from 0.18 gm. of sodium in specially dried absolute ethanol, and 1.70 gm. of ethyl acetamidomalonate and 1.2 ml. of dimethyl sulfate were added as described (5). Yield, 2.10 gm. (82 per cent); m.p., 131–133.5°.

The ester, 2.10 gm., was refluxed for 3 hours with a solution of 1.20 gm.

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[1] Mallinckrodt Chemical Works reagent grade copper nitrate gave better yields of formaldehyde than any of five other samples tested.

of sodium hydroxide in 10 ml. of water, and on cooling and acidification 1.57 gm. (90 per cent) of the malonic acid, m.p. 135–138° (with decomposition), were obtained. When this compound was heated to 140°, smooth decarboxylation occurred, and the acetyltryptophan so obtained was recrystallized from an alcohol-water mixture containing a trace of sodium hydrosulfite to give a yield of 92 per cent, m.p. 206–208°. Hydrolysis was accomplished by heating 0.53 gm. for 4 hours under nitrogen with 6 ml. of 2 N sulfuric acid. About 30 ml. of water were added, and the solution was heated, decolorized with charcoal, and neutralized to phenolphthalein with barium hydroxide. The hot solution was filtered free of barium sulfate and evaporated to dryness. The resulting tryptophan was recrystallized almost quantitatively from 15 ml. of acetic acid and 15 ml. of benzene, and 0.54 gm. (82 per cent) of glistening, colorless plates of tryptophan acetate salt containing a molecule of acetic acid of crystallization was obtained. This represents an over-all yield of 19 per cent based on carbon dioxide. The specific activity was 132,000 counts per minute per mg. (approximately 1 μc. per mg.).

Analysis—$C_{15}H_{20}O_6N_2$. Calculated. C 55.52, H 6.15
Found. " 55.52, " 6.12

Indole-3-acetic Acid-α-C^{14}—Gramine, 50 mg., and potassium cyanide, 38 mg., were dissolved in 1 ml. of 50 per cent ethanol, 0.1 ml. of dimethyl sulfate was added, and the mixture was refluxed for 1½ hours. The alcohol was then distilled, and the crude nitrile separated as a yellowish oil, which was extracted with ether. After evaporation of the ether, the nitrile was refluxed for 48 hours with 50 mg. of potassium hydroxide dissolved in 2.5 ml. of ethanol with 10 drops of water. The alcohol was then evaporated, and more water was added. The solution was extracted with ether to remove a small amount of nitrile and then acidified. The indole acetic acid thus obtained was recrystallized from water. Yield, 25 mg. (50 per cent, m.p. 163–165°); specific activity, 130,000 counts per minute per mg.

Analysis—$C_{10}H_9O_2N$. Calculated. C 68.56, H 5.18
Found. " 68.32, " 5.29

The acid was then converted to the sodium salt for storage.

DL-*Tryptophan-3-C^{14}*—This ring-labeled tryptophan was prepared in very low yield by the following series of reactions, in which no special modifications were made in the standardized procedures.

$$C_6H_5\overset{*}{C}OOH \xrightarrow[94\%]{} C_6H_5\overset{*}{C}OOC_2H_5 \xrightarrow[73\%]{} C_6H_5\overset{*}{C}H_3 \xrightarrow[58\%]{}$$

$$o\text{- and }p\text{-nitrotoluenes} \longrightarrow o\text{- and }p\text{-aminotoluenes} \xrightarrow[65\%]{}$$

$$o\text{- and }p\text{-formylaminotoluenes} \xrightarrow[33\%]{} \text{indole-3-}C^{14} \rightarrow \text{tryptophan-3-}C^{14}\text{ as before}$$

Carboxyl-labeled benzoic acid, 10 gm., was esterfied by the azeotropic method (8), and the ethyl benzoate was hydrogenated to toluene at 250° over copper-chromite catalyst (9). The toluene was nitrated in sulfuric acid solution (10), and the mixture of *o*- and *p*-nitrotoluene was hydrogenated in benzene solution at 210° over a nickel on kieselguhr catalyst (11). The crude amines were refluxed with 90 per cent formic acid and converted into indole (0.39 gm.), by treatment with potassium *tert*-butoxide followed by pyrolysis (12). Only the ortho isomer gave indole. The over-all yield of indole from benzoic acid was 8.5 per cent. The indole was then converted into tryptophan, with non-isotopic formaldehyde, by the series of reactions used for the β-labeled compound and in substantially the same yield. The specific activity was 6800 counts per minute per mg.

SUMMARY

1. DL-Tryptophan-β-C^{14}, indole-3-acetic acid-α-C^{14}, and DL-tryptophan-3-C^{14} have been synthesized.

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TRYPTOPHAN METABOLISM

I. CONCERNING THE MECHANISM OF THE MAMMALIAN CONVERSION OF TRYPTOPHAN INTO KYNURENINE, KYNURENIC ACID, AND NICOTINIC ACID*

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One of the first metabolic products of tryptophan to be reported was kynurenic acid, which was isolated by Ellinger in 1904 (1) from the urine of dogs that had been fed the amino acid. He characterized the compound correctly except for the assignment of the carboxyl group to the 3 position in the quinoline ring, but its correct location at the 2 position was determined soon afterward (2). Another apparent metabolic product, kynurenine, was discovered in 1931 by Kotake and Iwao (3), who assigned an incorrect structure to this molecule. In 1943, however, Butenandt *et al.* (4) determined its structure and confirmed it by synthesis. A third metabolite, xanthurenic acid, was isolated from the urine of pyridoxine-deficient rats that had previously been fed tryptophan (5).

Krehl *et al.* (6) in 1945 showed that rats would grow rapidly on low nicotinic acid diets containing corn, provided that additional tryptophan was added, and called attention to the possibility of a metabolic interrelation of this amino acid and nicotinic acid. They suggested that these results might be due at least in part to the intestinal flora. Shortly afterwards Rosen, Huff, and Perlzweig (7) demonstrated an increased excretion of nicotinic acid and N-methylnicotinamide in the urine of rats following the administration of tryptophan and suggested that tryptophan is converted into nicotinic acid. Since that time this conversion has been found to take place in humans (8) and several other mammals. A first understanding of the mechanism of this transformation resulted from the work of Beadle, Mitchell, and Nyc (9), who demonstrated that kynurenine is an intermediate in the conversion of tryptophan into nicotinic acid in mutant strains of *Neuro-*

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* This paper is based on work performed under contract No. W-7405-eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley.

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spora. It seemed desirable to ascertain whether this same mechanism might apply to mammals as well and to gain further definite evidence as to the exact nature of the conversion of tryptophan into kynurenine and kynurenic acid. Accordingly, the experiments with isotopic tryptophan (10) were undertaken, and a preliminary summary of our results has already been reported (11). During the course of this work, Mitchell and Nyc reported that 3-hydroxyanthranilic acid is also an intermediate in the conversion of tryptophan into nicotinic acid in *Neurospora* (12), and they obtained evidence from dietary experiments (13) that the compound also participates in the sequence of reactions in the rat. Further confirmation was afforded by the experiments of Albert, Scheer, and Deuel (14), who demonstrated increased excretion of N-methylnicotinamide in the urine of rats that had been given the hydroxyanthranilic acid. Kotake apparently has obtained independent evidence for these metabolic pathways.[1]

We are grateful to Mrs. Martha Kirk and Mrs. Olga Nave for technical assistance.

EXPERIMENTAL

Isolation of Kynurenine

A young, mature rabbit was maintained for 3 weeks on a diet of polished rice, cooked in a minimum of water. On this régime, the animal lost 25 per cent of its weight. A suspension of 4.00 gm. of DL-tryptophan-β-C^{14} in isotonic saline (30 ml.) was then injected subcutaneously. The urine was collected under toluene, and the L-kynurenine sulfate was isolated by slight modifications of the method of Butenandt *et al.* (4). The 48 hour urine which gave a strong qualitative test for kynurenine was acidified with sulfuric acid and filtered. The filtrate was concentrated at room temperature to one-fifth of its volume; ethanol, sufficient to make an 80 per cent solution, was added, and the solution was allowed to stand for 2 days in the refrigerator. At the end of this time, the crystalline kynurenine was filtered and recrystallized from dilute ethanol. The kynurenine sulfate which still contained considerable inorganic impurities was converted into the acetate salt by crystallization from acetic acid-benzene, and 350 mg. of an analytically pure sample were obtained. Its specific activity was determined by combustion.

The specific activity of administered tryptophan was 308 counts per minute per mg.; the specific activity of kynurenine acetate was 113 counts per minute per mg. If the entire dose of tryptophan were converted directly into kynurenine, its specific activity would have been 303 counts per minute per mg. Therefore, there was a dilution of 63 per cent by the body pool of tryptophan and kynurenine.

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[1] Private communication from Dr. R. Kinoshita.

Kynurenine Degradation

The kynurenine acetate thus obtained was treated with sodium hypoiodite, and iodoform of correct specific activity was obtained, indicating that only the β-carbon atom of the compound contained the label. This reaction involves the oxidation of the α-amino acid group with the loss of carbon dioxide and ammonia to give *o*-aminobenzoylacetaldehyde (15) which is then cleaved in alkali in either of the two possible ways, each of which would give iodoform derived from the same carbon atom on reaction with another molecule of hypoiodite.

The labeled kynurenine acetate, 3.2 mg., was diluted with 50.4 mg. of carrier kynurenine acetate, and after one recrystallization its specific activity was determined by the direct plating technique (16) and found to be 6.8 counts per minute per mg. This sample, 35 mg., was boiled for 1 hour with N sodium hydroxide, then cooled, and a solution of sodium hypoiodite was added. The iodoform, which separated immediately, was centrifuged, washed with water, and recrystallized from dilute ethanol. The specific activity, determined by direct plating, was 4.7 counts per minute per mg. (calculated, 4.8 counts per minute per mg.).

Kynurenic Acid Isolation

Two young male dogs of pure bred cocker spaniel stock were maintained on a stock diet (17), and each was given a solution of 500 mg. of DL-tryptophan-β-C^{14} in 5 ml. of water containing enough hydrochloric acid to dissolve the amino acid. The urines were collected under toluene for 36 hours and then pooled and acidified with sulfuric acid. A blackish precipitate of crude kynurenic acid was obtained by centrifugation and clarified three times with charcoal in an alkaline solution, followed by acidification. The light yellow crystalline solid was then recrystallized twice from dilute acetic acid, and 150 mg. of pure kynurenic acid was obtained.

Analysis—$C_{10}H_7O_3N$. Calculated, C 63.5, H 3.7; found, C 63.2, H 3.9

The specific activity of the ingested tryptophan was 460 counts per minute per mg., that of kynurenic acid, 143 counts per minute per mg. If the administered tryptophan had been converted directly into kynurenic acid, its specific activity would have been 496 counts per minute per mg. Therefore, it was diluted 71 per cent by the body pool of tryptophan and kynurenine.

Kynurenic Acid Degradation

The position of the labeled carbon atom in the radioactive kynurenic acid was established with considerable certainty by use of a series of reactions described in the literature (18). Kynurenic acid when heated to 300° undergoes smooth decarboxylation, and 4-hydroxyquinoline is obtained. When this reaction was carried out on the labeled compound, the evolved carbon dioxide was devoid of radioactivity, proving that the label is not in the carboxyl group.

The kynurenic acid, (I) 20 mg., was treated for 5 hours at 55° with a solution containing 10 mg. of potassium hydroxide and 64 mg. of potassium permanganate in 3 ml. of water. The manganese dioxide was removed by centrifugation, and the excess permanganate was decolorized by the addition of a few drops of sodium sulfite. The colorless solution was acidified, and colorless needles of *o*-carboxyoxanilide (II) were obtained, which recrystallized from hot water to give 7.4 mg. of purified product, specific activity 127 counts per minute per mg. (calculated, 130).

The tagged compound, 2.01 mg. with 30.7 mg. of carrier, was refluxed for ½ hour with 2 ml. of 2.5 N hydrochloric acid, 100 mg. of anhydrous calcium chloride in 2 ml. of water were added, and the solution was taken to pH 8 with ammonium hydroxide. The calcium oxalate was filtered, washed with alcohol and ether, and plated directly. Yield, 15.5 mg. (87 per cent); specific activity, 11.6 counts per minute per mg. (calculated, 12.2). This proves that the label is in the side chain of the *o*-carboxyoxanilide and must have been derived from positions 2 or 3 of the kynurenic acid.

When heated above its melting point, *o*-carboxyoxanilide evolves carbon dioxide and carbon monoxide, and the radioactivities of these gases were determined. A mixture of 3.79 mg. of active and 42.4 mg. of carrier oxanilide was placed in a small flask and heated to 215°. The effluent gases were swept with nitrogen through a sodium hydroxide bubbler, then through a tube filled with copper oxide at 600°, and finally through a second sodium hydroxide bubbler. The carbon dioxide was absorbed first, and the carbon monoxide was oxidized to carbon dioxide in the combustion tube, trapped in the second bubbler, and then precipitated as barium carbonate. The barium carbonate derived from the evolved carbon dioxide weighed 34.5 mg. (80 per cent corrected for the blank) and had a specific activity of 10.6 counts per minute per mg. (calculated, 10.5). The barium carbonate derived from the carbon monoxide weighed 12.5 mg. (29 per cent) and was completely without radioactivity. This proves that only one of the two possible carbon atoms in the kynurenic acid was labeled. It seemed most likely that the carbon dioxide obtained during the decomposition of the *o*-carboxyoxanilide (II) arose from the carboxyl group, and the carbon monoxide from the carbonyl group. If this were true, the intermediate formed after the initial loss of carbon dioxide would be formylanthranilic acid (III), which should liberate carbon monoxide when heated. This hypothesis was tested by the use of a synthetic sample, and a 34 per cent yield of carbon monoxide was obtained. This is in fair agreement with the 38 per cent that would be predicted on the basis of the results obtained from the decomposition of the *o*-carboxyoxanilide. Thus it is extremely probable that only the 3 position in kynurenic acid was labeled.

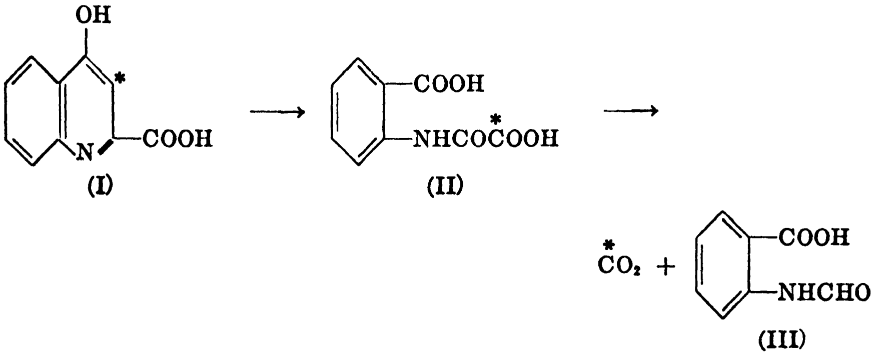


TABLE I

Radioactivities and Fluorometric Data

	N-Methyl-nicotinamide, total	Total counts per min.	Specific activity, counts per min. per mg.
	γ		
Tryptophan administered		1,020,000	4900
Urine (before tryptophan feeding)	42	0	
" (after " ")	450	7,400	
Permutit filtrate	0	4,200	
Permutit-KCl eluate	115	210	
N-Methylnicotinamide picrate	Carrier	0	0

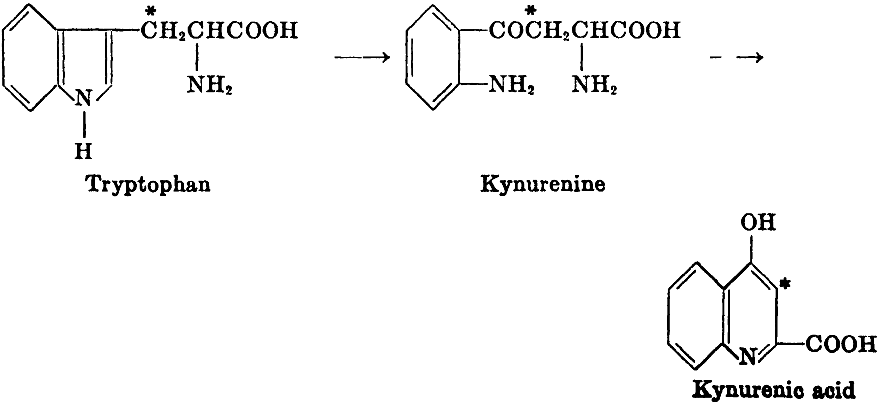
N-Methylnicotinamide Experiment

Three young male rats (average weight, 300 gm.) of the Department of Home Economics stock colony were maintained for 1 week on a nicotinic acid-deficient diet (19) at the 12 per cent casein level, an aqueous solution of 65 mg. of DL-tryptophan-β-C^{14} was given each by stomach tube, and their urines were collected under toluene for 36 hours and pooled. The N-methylnicotinamide was isolated by the procedure of Hochberg, Melnick, and Oser (20) and followed fluorometrically by the method of Huff and Perlzweig (21). The urine was diluted with acetate buffer at pH 4.5, and the mixture was then passed over a permutit column. The N-methylnicotinamide was then eluted with hot potassium chloride solution. Both filtrate and eluate were collected. Each step was followed by fluorometric analysis for N-methylnicotinamide and by radioactivity measurements. The results are shown in Table I. To the potassium chloride eluate were added 50 mg. of N-methylnicotinamide chloride as carrier, and the solution was concentrated to dryness in a vacuum. The residue was extracted several times with boiling 95 per cent ethanol, and the filtered extract was evaporated to dryness and reextracted with ethanol. The solution was concentrated to 3 ml., and several drops of a saturated solution of picric acid in ethanol were added. The picrate salt of N-methylnicotinamide was obtained (35 mg., m.p. 178–183°), which was recrystallized from ethanol. The melting point was 187–188° and was not depressed on admixture with an authentic sample. A direct plate was made from 12.3 mg. of this purified picrate, and no radioactivity could be detected even when it was placed in the nucleometer. If the radioactive carbon atom had been retained in this conversion, there would have been 414 counts per minute on the plate.

A similar experiment was carried out in which the labeled kynurenine, obtained from rabbit urine, was administered to nicotinic acid-deficient rats. Although the N-methylnicotinamide excretion was increased from 145 to 730 γ, there was no detectable radioactivity in the picrate isolated as described.

DISCUSSION

The fact that kynurenine and kynurenic acid, isolated from the urines of animals that had been given labeled tryptophan, were radioactive proves that the conversion of the amino acid into these products had actually taken place. The lowered specific activities of the products indicate considerable dilution of the administered dose by the body pools of tryptophan and the other intermediates. The demonstration that the β-carbon atom of tryptophan becomes the β-carbon of kynurenine proves that the change takes place by only one possible mechanism, which is indicated in the formulas, and which is identical with that previously demonstrated in *Neurospora* (9). In addition the ring closure of kynurenine to kynurenic acid is clearly demonstrated for the first time, as shown in the accompanying scheme.



It is evident that the indole ring becomes oxidized, possibly through the intermediate of 2-hydroxytryptophan. The next product of the oxidation might then be formylkynurenine, which would immediately be hydrolyzed in the body to kynurenine. The conversion of kynurenine to kynurenic acid might be formulated by the oxidative deamination of the α-amino group in kynurenine (another example of this well known biochemical transformation), followed by an intramolecular condensation between the *o*-amino group and the α-keto group to give the quinoline derivative, kynurenic acid. It had seemed possible that kynurenic acid might further be oxidized to give nicotinic acid, an attractive hypothesis because of the pyridine ring already present in the molecule. If this were the case, the nicotinic acid isolated would have been radioactive. The lack of radioactivity of the N-methylnicotinamide picrate shows that under the conditions of our experiments less than 0.005 per cent of the observed conversion could have taken place by such a mechanism. Therefore the side chain of tryptophan must be lost during the transformation. We have no direct evidence in these experiments for the participation of 3-hydroxyanthranilic acid as an intermediate, but our results are certainly compatible with such a mechanism.

SUMMARY

1. Experiments with radiocarbon prove that DL-tryptophan is converted into kynurenine in rabbits and kynurenic acid in dogs.

2. The β-carbon atom of the tryptophan becomes the β-carbon of kynurenine and the 3-carbon atom of kynurenic acid. Thus the conversion takes place by only one mechanism.

3. The administered dose of tryptophan is considerably diluted by the body pool of this substance and other intermediates.

4. The side chain of tryptophan is lost during its conversion into nicotinic acid.

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TRYPTOPHAN METABOLISM

II. CONCERNING THE MECHANISM OF THE MAMMALIAN CONVERSION OF TRYPTOPHAN INTO NICOTINIC ACID*

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Although the conversion of tryptophan into nicotinic acid had been demonstrated by numerous nutrition experiments for the past several years, relatively little was known about the mechanism of this change until the recent studies by Mitchell and Nyc (1) on mutant strains of *Neurospora* proved that 3-hydroxyanthranilic acid is a key intermediate in the metabolic sequence. That the same compound is involved in the mammalian conversion is indicated by the feeding experiments of Mitchell, Nyc, and Owen (2), who reported that it could maintain growth of rats on a nicotinic acid-deficient diet, and by Albert, Scheer, and Deuel (3) who found an increased excretion of N-methylnicotinamide by rats fed this substance. In the isotopic experiments (4) the fact that the labeled β-carbon atom of tryptophan did not appear in the nicotinic acid proved that the side chain is lost in the transformation, and this observation is also consistent with the existence of 3-hydroxyanthranilic acid as an intermediate. However, since we had not demonstrated by tracer experiments the conversion of tryptophan into nicotinic acid, a differently labeled tryptophan-3-C^{14} was synthesized (5) so that this point might be proved. It was also hoped that further light might be shed on the rather obscure mechanism of the transformation of 3-hydroxyanthranilic acid into nicotinic acid.[1]

We wish to thank Dr. A. F. Morgan for her generosity in making the facilities of her laboratory available for these investigations. We are grate-

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‡ On leave from the Sir William Dunn School of Pathology, University of Oxford, England.

[1] In the preliminary account of this work (6), the asterisk in the tryptophan formula was incorrectly represented as being over the β- instead of the 3-carbon atom.

ful to Mrs. Olga Nave, Mrs. Martha Kirk, and Mrs. Yvonne Stone for technical assistance, and deeply indebted to Mrs. Mary E. Gullberg and Miss Dorothea Maybee who generously volunteered their services for these experiments.

TABLE I

Fluorometric Data

	N-Methylnicotinamide, total
	γ
Urine before tryptophan feeding	38
" after " "	790
Permutit-KCl eluate	175

TABLE II

Radioactivities

	Total counts per min.	Specific activity, counts per min. per mg.	
		Found	Calculated
Tryptophan administered	620,000	2740	
Urine	18,800		
N-Methylnicotinamide picrate	79	0.75	3.5*
Nicotinic acid	70	2.0	2.2
Barium carbonate from nicotinic acid decarboxylation	21	1.3	1.2

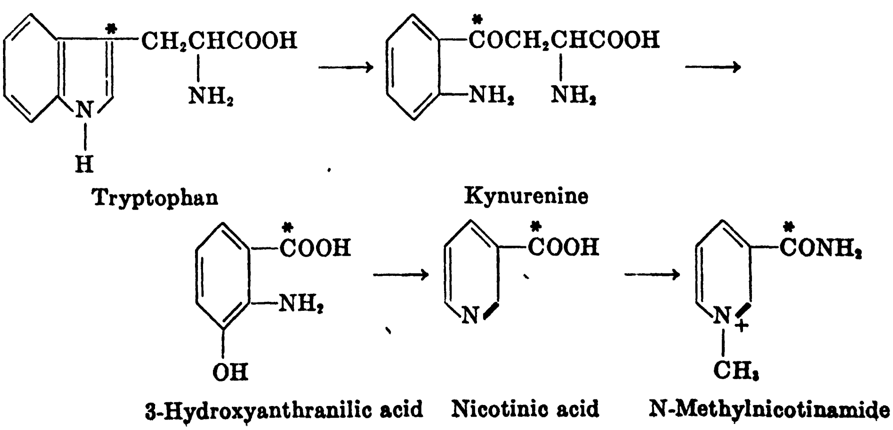
* This figure is calculated as follows. Assuming the direct conversion of the administered dose and the specific activity of tryptophan to be equal to 2.74 counts per minute per microgram, then the specific activity of N-methylnicotinamide chloride would be 3.25 counts per minute per microgram. Since there were 175 γ in the eluate, there would be $3.25 \times 175 = 570$ counts per minute total. Now, 76 mg. of carrier were added, so that the specific activity of the N-methylnicotinamide chloride would be 7.5 counts per minute per mg., which is equivalent to 3.5 counts per minute per mg. of the picrate. Thus, there was a 5-fold dilution by the body pools of tryptophan and nicotinic acid (and the various intermediates) in this experiment.

EXPERIMENTAL

Three young, male rats were fed for a week with a 12 per cent casein-niacin-deficient diet (7) and were then each given by stomach tube a solution of 75 mg. of DL-tryptophan-3-C^{14} in 2 ml. of water. The urines were collected under toluene for 36 hours and pooled. The N-methylnicotinamide was determined fluorometrically (8) throughout the experiment, and the results are indicated in Table I. The radioactivities were also followed, and these results are shown in Table II. These measurements were carried out with an internal proportional counter (nucleometer) by means of the direct plating technique (9). The N-methylnicotinamide isolation was carried out according to a known procedure (10).

The urine (16 ml.) was collected; 14 ml. were diluted to 50 ml. with acetate buffer at pH 4.4 and passed through a permutit column. Fluorometric analysis indicated that no N-methylnicotinamide was present in the filtrate. The compound was eluted from the column by washing with 15 ml. of hot 25 per cent potassium chloride, followed by 125 ml. of water. Carrier N-methylnicotinamide chloride (76 mg.) was added to the eluate, and the mixture was evaporated to dryness in a vacuum. The residue was extracted with three 50 ml. portions of 95 per cent ethanol, and the extract again evaporated to dryness. The residue was then reextracted with 95 per cent ethanol and centrifuged clear. After addition of a solution of picric acid in ethanol to the extract, a precipitate of the picrate of N-methylnicotinamide was obtained, which was recrystallized twice from 90 per cent ethanol. The purified picrate, 106 mg., m.p. 187–188°, gave no depression of the melting point on admixture with an authentic sample.

The radioactive picrate, 105 mg., was converted into the corresponding chloride by solution in dilute hydrochloric acid, followed by extraction of the free picric acid with ether. The N-methylnicotinamide chloride was heated with 1.5 ml. of concentrated hydrochloric acid in a sealed tube for 27 hours at 250°. The resulting solution was evaporated to dryness, the residue was dissolved in 3 ml. of water, and the solution was made slightly alkaline with sodium hydroxide. This solution was then boiled until the evolution of ammonia ceased, neutralized, and mixed, while hot, with 60 mg. of copper sulfate in 0.5 ml. of water. The mixture was cooled, and the copper salt of nicotinic acid was filtered and washed with cold water. The salt was suspended in 5 ml. of water, and decomposed with hydrogen sulfide. Evaporation of the solution gave 35 mg. of nicotinic acid, m.p.



218–220° (uncorrected). This melting point was not raised by recrystallization of the compound from moist butanol, nor was it depressed on admixture of an authentic sample of nicotinic acid.

The radioactive nicotinic acid, 13.4 mg., was heated with quinoline and copper-chromite catalyst (11) to 285°. The carbon dioxide resulting from the decarboxylation was swept with pure nitrogen into a bubbler containing sodium hydroxide and was then precipitated as barium carbonate. The specific activity of the barium carbonate indicates that the entire radioactivity of the nicotinic acid was present in the carboxyl group.

DISCUSSION

These tracer experiments together with the ones with tryptophan-β-C^{14} (4) prove that the conversion of tryptophan into nicotinic acid in the intact mammal takes place by a mechanism identical to that already demonstrated to occur in *Neurospora*.

The conversion of tryptophan into kynurenine had been demonstrated, and evidence is presented in the preceding paper that this occurs directly and by only one mechanism. Now it has been shown that carbon atom 3 in the indole ring of tryptophan becomes the carboxyl carbon of nicotinic acid. (Throughout this work we have referred to nicotinic acid as the end-product of this sequence of reactions, although we have in all cases isolated N-methylnicotinamide. Hundley and Bond (12) have shown in experiments with C^{13} that nicotinic acid is excreted almost quantitatively as its N-methyl amide.) Although we have no direct evidence for the formation of 3-hydroxyanthranilic acid in these experiments, our results are consistent with its participation in the metabolic sequence and there can be little doubt from the work of others that it is an intermediate.

It is clear that the tagged indole 3-carbon atom is the precursor of the carboxyl carbon of the hydroxyanthranilic acid, so that it has now been demonstrated that the carboxyl group of hydroxyanthranilic acid becomes the carboxyl group of nicotinic acid. Although the exact mechanism of this conversion of a benzene into a pyridine derivative still remains to be elucidated, this observation should be of some importance in the final solution of the problem. It seems rather likely that the benzene ring opens, carbon atom 3 which carried the hydroxyl group is lost, and that ring closure is effected somehow between carbon atom 2 and the nitrogen atom. However, the details of these reactions remain to be ascertained.

SUMMARY

1. The conversion of tryptophan into nicotinic acid in the rat has been established with certainty by isotopic experiments.
2. The mechanism of this change is identical with that previously shown

for *Neurospora* and consists of a sequence in which the principal compounds are tryptophan, kynurenine, 3-hydroxyanthranilic acid, and nicotinic acid.

3. Carbon atom 3 in the indole ring of tryptophan, the precursor of the carboxyl carbon atom of the 3-hydroxyanthranilic acid, becomes the carboxyl carbon atom of the nicotinic acid.

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A FLUOROMETRIC METHOD FOR THE DETERMINATION OF THE 6-PYRIDONE OF N^1-METHYLNICOTINAMIDE IN URINE*

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(Received for publication, January 10, 1949)

Since Knox and Grossman demonstrated the excretion by man of the 6-pyridone of N^1-methylnicotinamide (hereafter referred to as the pyridone) in appreciable amounts after the ingestion of nicotinamide (2), it became necessary to devise a method for its quantitative estimation in urine. Knox and Grossman isolated the compound from human urine and described its properties, including its ultraviolet absorption spectrum. They found that this metabolite did not yield a fluorescent derivative under the conditions employed for the determination of N^1-methylnicotinamide (3); nor could it be determined microbiologically with the use of the customary organisms.

The above observation by Knox and Grossman was confirmed that the condensation reaction with acetone in an aqueous alkaline medium which yields a fluorescent product with N^1-methylnicotinamide and the pyridone nucleotides (3) did not yield a fluorescent compound with the pyridone. It was soon found, however, that a fluorescent compound could be obtained by agitating the pyridone in acetone solution with KOH in the presence of a minimal amount of water. With the applicability of this reaction to analytical purposes in pure solutions of the pyridone established, it was then necessary to find a method of separation and purification of relatively small amounts of the pyridone present in normal human urine which would yield reasonable recovery and blank values. This proved to be extremely difficult. The usual adsorption and elution techniques either were altogether inapplicable or gave variably low recovery values. The final resolution of this difficulty was found in two steps: the removal of pigments and of other interfering substances in urine with lead

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† Nutrition Foundation, Inc., Fellow. The data of this paper are taken from the dissertation for the degree of Doctor of Philosophy to be submitted by F. Rosen to Duke University, 1949.

subacetate and the extraction of the pyridone from the filtrate, saturated with K_2CO_3, with ether. This method of separation and extraction also permits the estimation of the pyridone spectrophotometrically.[1]

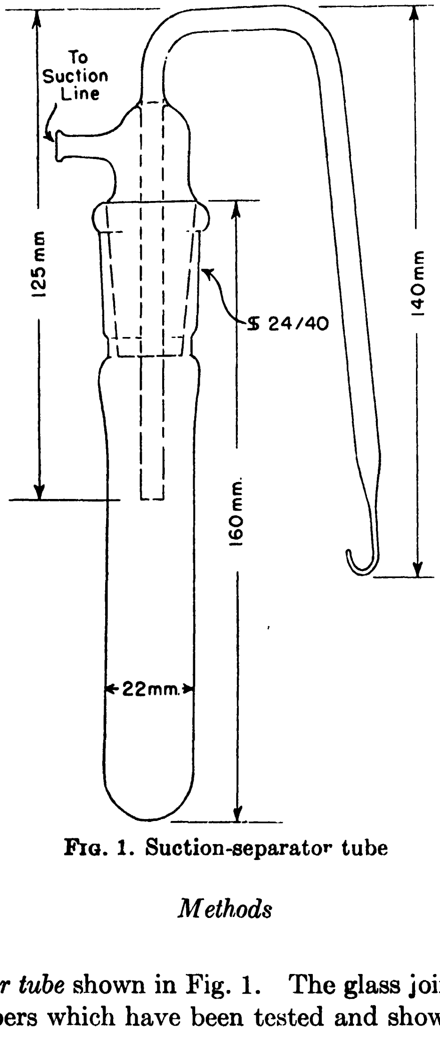

FIG. 1. Suction-separator tube

Methods

Apparatus—

Suction-separator tube shown in Fig. 1. The glass joint may be replaced by neoprene stoppers which have been tested and shown to contribute no fluorescent material to the ether extract. For equal evaporation of the ether in all the tubes, the opening at the tip of the separator should be approximately 0.25 mm. in diameter.

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[1] The authors acknowledge their indebtedness to Dr. W. E. Knox and Dr. K. L. Pines for communicating certain details of their preliminary procedure for the separation of the pyridone in urine by adsorption on Lloyd's reagent, followed by elution and spectrophotometric estimation.

22 × 150 mm. glass-stoppered test-tubes, obtained from the Scientific Glass Apparatus Company, Bloomfield, New Jersey.

Fluorometer cuvettes. A set of calibrated Pyrex test-tubes is selected for the Coleman (18 × 150 mm.) and Farrand (10 × 75 mm.) fluorometers. The tubes are calibrated with an appropriate solution of quinine sulfate in the sensitivity range to be used.

0.1 ml. micro pipette graduated in 0.01 ml. The tip of the pipette is constricted slightly and then bent at a 45° angle to facilitate the addition of the 12 N KOH. It is advisable to attach a piece of rubber tubing to the mouth of the pipette when the strong alkali is being drawn up.

Test-tube racks to hold forty to 50 tubes. Such racks may be home-made from 1¾ inch wood boards through which holes of uniform diameter are drilled. A smooth piece of thin plywood is nailed on to provide the bottom. For the 10 mm. Farrand cuvette tubes the holes should be 15 mm. in diameter and so spaced as to prevent the tubes touching each other when shaken. Rubber-plated metal serological Kahn tube racks, Sargent catalogue No. S-7935, can be conveniently used for this purpose. Care must be taken to provide uniform shaking to all tubes in any single run, and also to prevent the scratching of the surface of the tubes by abrasion in the rack.

Shaking machine. For this procedure we have employed a Kahn shaker which makes about 280 excursions per minute (A. S. Aloe Company, catalogue No. 14680).

Parafilm.[2]

Photofluorometers. This method was developed largely with the use of the new type of sensitive fluorometer described by Lowry (4) and manufactured by the Farrand Optical Company. This instrument is based on the use of a multiplier phototube, and is capable of measuring fluorescence over a very wide range of sensitivity with a hitherto unobtainable degree of stability of readings. The method was also adapted for use with the Coleman model 12A and the Lumetron (Photovolt Corporation) fluorometers, as described in the text below.

Reagents—

Saturated lead subacetate solution (6 gm. made to 100 ml. with water) (prepared from Merck's reagent grade, "suitable for sugar analysis, by W. D. Horne's method").

12 N potassium hydroxide solution; U. S. P. and reagent grades were found satisfactory.

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[2] Parafilm is obtainable from the Will Corporation, Rochester, New York, or the Southern Scientific Company, Atlanta, Georgia. The tubes may also be capped with pieces of ordinary wax paper reinforced with adhesive tape and fastened to the tubes with rings cut from rubber tubing.

Acetone, rendered free from fluorescent material by distillation over $KMnO_4$ and stored over anhydrous Na_2SO_4 in a brown bottle.

Ether. Impurities removed from commercial ether by washing once with a dilute solution of $FeSO_4$ and twice with water before distilling a few days supply.

Standard pyridone solution containing 300 γ per ml., made by dissolving 15 mg. of the pyridone[3] in 50 ml. of redistilled acetone. To prevent a change in concentration by the evaporation of acetone, it was found desirable to store the solution in a desiccator containing an open layer of acetone. This solution is stable for at least 6 weeks if kept in a cool room. Working standard solutions for the various fluorometers are prepared by diluting an appropriate amount of the stock standard to a given volume with acetone.

Quinine solution for standardization of fluorometers. Stock solution, 0.1 mg. per ml. of quinine sulfate in 0.1 N H_2SO_4. Working standard solution, 0.3 γ of quinine sulfate per ml. in 0.1 N H_2SO_4. For standardizing the Farrand (aperture No. 6) and Coleman fluorometers, 0.02 and 0.06 γ of quinine sulfate per ml. in 0.1 N H_2SO_4, respectively, are prepared fresh for daily use. All quinine solutions are kept in the dark.

Procedure

For use with the Farrand fluorometer, an aliquot of urine estimated to contain from 20 to 50 γ of the pyridone (*e.g.*, 2 to 4 ml. of a normal human urine) is neutralized to pH 6.5 to 7.5 and decolorized with a saturated solution of lead subacetate. To remove the pigments of human urine of specific gravity 1.028 or less, 1.3 volumes of the saturated lead subacetate solution to 1 volume of urine are required. For more concentrated urines, a ratio of 1.7:1 has been found adequate. When the concentration of pyridone is very low, 8 ml. of undiluted urine may be treated directly with about 250 mg. of solid lead subacetate. The total volume is brought to 8 ml. with water, shaken briefly, and filtered (Whatman No. 30) or centrifuged; 3.00 ml. of the filtrate are transferred to a 22 × 150 mm. glass-stoppered test-tube, 4 gm. of powdered K_2CO_3 are added, and the tube shaken to saturate the solution.

The saturated solution is extracted consecutively with four 10 ml. portions of washed and redistilled ether. For each extraction, the tube is tightly stoppered and shaken for 30 seconds. After a brief centrifugation

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[3] The 6-pyridone used in this study was synthesized by the recent method of Huff (5). The authors are indebted to Dr. J. W. Huff, of the Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pennsylvania, for supplying samples of this product for use in this study.

(20 seconds at moderate speed),[4] the ether layer is carefully removed with gentle suction into dry glass-stoppered receivers (see Fig. 1), which are immersed in warm water (40°) in 8 ounce bottles. If the suction is maintained during the period of extraction and the water in the bottles kept warm, the ether will usually be evaporated within 10 minutes after the last extraction.

A separate sample of urine to which 75 γ of pyridone have been added is treated in like fashion and serves to provide an internal standard. The 75 γ of pyridone contained in 1.0 ml. of acetone are added to a dry test-tube and the acetone completely evaporated with warming, in an air current provided by suction, before the addition of the urine. If acetone is present in the lead filtrate, a significant amount of fluorescent material will be extracted by the ether and the blank values will be extremely high.

After evaporation of the ether in the air current, the residue in the bottom should appear completely dry. If a noticeable amount of water persists, this indicates contamination with the aqueous phase, and the ether extraction should be repeated on another aliquot of the lead filtrate. To the dry residue in the tubes are added 10.0 ml. of dry acetone and a pinch of anhydrous Na_2SO_4. Into a series of fluorometer cuvettes (10 × 75 mm. tubes), carefully cleaned and dried by draining after rinsing with redistilled acetone, are measured 0.50 ml. aliquots of the above acetone solutions. To each tube are added, serially, 0.20 ml. of acetone and 0.010 ml. of 12 N KOH. The latter is added with utmost care by resting the curved tip of the pipette against the wall of the test-tube about 5 mm. above the surface of the acetone. The tubes are covered with a tight fitting cap made from a square piece of Parafilm.[2] The urine blank is obtained by adding 1.00 ml. of water to a tube prepared as above. Two reagent blanks are prepared as follows: Into each of two tubes are measured 0.70 ml. of dry acetone and 0.01 ml. of 12 N KOH. To one, 1.0 ml. of H_2O is added at once, the contents thoroughly mixed, and the tube permitted to stand along with the above urine blank tube without shaking. The second tube is capped and shaken along with the others, as described in the next paragraph. The use of these blanks will be explained in detail below.

The tubes are placed in a rack and shaken for 1 hour at 23° ± 2°. While at temperatures above 23° the fluorescence of the pyridone-acetone compound is increased, there is a simultaneous rise in the reagent blank which may cancel the advantage gained. Special care must be taken to avoid overheating all or some of the tubes by proximity to the motor of the shaker or to other sources of heat. This is best controlled by attaching thermometers to two ends of the rack holding the tubes.

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[4] The centrifugation of the glass-stoppered tubes accomplished either in a No. 2 International centrifuge equipped with 100 ml. tube shields or in a No. 1 centrifuge equipped with an angle head to hold 100 ml. shields.

To all the tubes that were shaken 1.0 ml. of water is added and the contents mixed at once. The urine blank and the reagent blank tubes, which were previously made up to 1.7 ml., are used without further dilution. The tubes are read in the Farrand fluorometer fitted with thiamine filters. A standard quinine solution containing 0.02 γ per ml. is used to set the fluorometer at the desired sensitivity (at 15 to 50 divisions on the galvanometer).

Example of Determination—A normal human 24 hour urine, volume 1000 ml., of specific gravity 1.018. Taken for analysis, 3.00 ml. are diluted to 8.00 ml. with 4.00 ml. of lead subacetate and 1.00 ml. of water; 3.00 ml. of filtrate are saturated with K_2CO_3 extracted with ether and evaporated; the residue is taken up in 10.0 ml. of acetone; 0.5 ml. of acetone solution is taken for analysis, containing $0.5/10 \times 3 \times 3/8 = 0.056$ ml. of the original urine.

The agreement of actual values obtained with triplicate aliquots from the same acetone extract of a urine, and of the same urine with added pyridone (recovery), is shown in Table I.

These data were obtained on two complete analyses of a normal human urine carried out on separate days, in each of which one sample of urine, with and without added pyridone, was subjected to the entire procedure.

It will be noted that within each of these determinations triplicate analyses of the same acetone extract and duplicate blank analyses agree within 0 to 1.0 galvanometer divisions. On the other hand the readings obtained in separate analyses on the various phases of the procedure (*i.e.*, urine, urine + pyridone, urine and reagent blanks) may diverge quite widely, and yet, as the calculations show, yield final values which agree reasonably well. This is due to the compensating effect of the various blank values and demonstrates the necessity for the use of the internal standard and of the four blank determinations with each analysis. The differences in the readings obtained in the separate analyses may be due to several factors, such as incomplete or excessive drying of the ether residues, contamination with external impurities from the air or from the aqueous phase in the separation of the ether, differences in the reagents, or in temperature, and humidity changes.

Other analyses repeated on the same urines on different days yielded the following results, in mg. per 24 hours (the higher values were obtained after ingestion of large amounts of nicotinamide): 5.4, 5.9; 9.2, 9.5; 221, 194; 282, 274; and 337, 356.

On the basis of the above considerations it may be recommended that, after the initial period of practice, urine analyses be carried out on single

TABLE I

Values Obtained with Triplicate Aliquots from Urine

Tube No.	Contents	Analyses on			
		Dec. 19, 1948		Dec. 21, 1948	
		Galvanometer* divisions	Average	Galvanometer divisions	Average
1	0.5 ml. urine filtrate	26		26	
2	0.5 " " "	26.5		25.5	
3	0.5 " " "	27	26.5	25.5	25.7
4	0.5 " " " + 1.0 ml. H_2O	14		12	
5	0.5 ml. urine filtrate + 1.0 ml. H_2O	14	14	12	12
6	0.5 ml. urine filtrate + 1.4 γ pyridone	51.5		46.5	
7	0.5 ml. urine filtrate + 1.4 γ pyridone	52		46.5	
8	0.5 ml. urine filtrate + 1.4 γ pyridone	51.5	51.7	47	46.6
9	0.5 ml. urine filtrate + 1.4 γ pyridone + 1.0 ml. H_2O	16		12	
10	0.5 ml. urine filtrate + 1.4 γ pyridone + 1.0 ml. H_2O	16	16	12	12
11	1.0 ml. acetone	6.5		8	
12	1.0 " "	7.0	6.7	8	8
13	1.0 " " + 1.0 ml. H_2O	3		2.5	

* Galvanometer of Farrand fluorometer, set at 30 divisions with 0.02 γ per ml. of quinine sulfate standard.

Calculations

Analysis on December 19, 1948

$(51.5 - 16) - (26.5 - 14) = 23$ divisions for 1.4 γ of added pyridone

$\frac{23}{1.4} = 16.5$ divisions for 1.0 γ pyridone

$7 - 3 = 4$ divisions due to acetone + KOH product

$26.5 - 14 - 4 = 8.5$ divisions due to pyridone in 0.056 ml. of urine

$\frac{8.5}{16.5} \times \frac{1000}{0.056} = 9.2$ mg. of pyridone in 24 hour urine

Analysis on December 21, 1948

$(46.5 - 12) - (25.5 - 12) = 21$ divisions for 1.4 γ pyridone

$\frac{21}{1.4} = 15$ divisions for 1.0 γ pyridone

$8 - 2.5 = 5.5$ divisions due to acetone + KOH product

$25.5 - 12 - 5.5 = 8$ divisions due to pyridone in 0.056 ml. of urine

$\frac{8}{15} \times \frac{1000}{0.056} = 9.5$ mg. of pyridone in 24 hour urine samples with and without added pyridone (internal standard). The recovery value for the internal standard should furnish the criterion as to the reliability of the results; recovery values below 75 per cent of the theoretical indicate the desirability of repeating the analysis. In this connection it may be desirable to include at frequent intervals a duplicate pair of tubes containing an appropriate amount of the standard solution and of its water blank.

Modification for Coleman Model 12A Fluorometer—An aliquot of urine containing 50 to 100 γ of pyridone is treated, as described above, with lead, extracted with ether, evaporated, and the residue dissolved in 10 ml. of acetone. Into cuvettes (18 × 150 mm. test-tubes) are then measured 1.00 ml. of the acetone solution (containing 2 to 4 γ of pyridone) and 0.01 ml. of 12 N KOH, the tubes are capped, and the reaction mixture shaken for 1 hour at 23° ± 2°. All urine blanks are obtained by adding 1.0 ml. of water to the appropriate tubes and the reagent blank prepared with 1.0 ml. of acetone. After shaking, the tubes are diluted to a total volume of 8 ml. with 5 ml. of redistilled acetone and 2 ml. of water. The fluorescent compound is measured in the fluorometer standardized at 100 galvanometer divisions with a solution of quinine sulfate (0.06 γ per ml.). For the internal standard (recovery) the same procedure is followed simultaneously for another sample of urine to which 75 γ of the standard pyridone had been added.

Use of Lumetron Fluorometer (Model EF2)—This instrument is considerably less sensitive than the others and employs an adapter for 22 mm. test-tubes as cuvettes and the galvanometer as a direct reading instrument. It was found that 20 to 50 γ of the pyridone present in 13 ml. of the final solution were required for reproducible results. Such amounts may be easily obtained by appropriate dilutions of human urines after doses of niacin, but are not obtainable with normal control urines, unless considerably larger amounts are extracted.

The reaction mixture consists of 1.0 ml. of the acetone filtrate (dry residue from ether may be dissolved in 5 ml. of acetone) and 0.01 ml. of 12 N KOH. Urine blanks are prepared with 1.0 ml. of water. In the sensitivity range measured by the Lumetron, the acetone reagent blank is zero and may be omitted after initial checking. To increase further the fluorescence of the pyridone-acetone compound for use with this fluorometer, the tubes are shaken for 1½ hours (or longer) and then diluted to 13 ml. (minimal volume required for the test-tube adapter) with 7 ml. of acetone and 5 ml. of water.

The technique above for the isolation of the pyridone from urine by means of ether extraction from a lead subacetate filtrate lends itself advantageously to the *spectrophotometric estimation* of the substance. The residue, after the evaporation of the ether, is dissolved in a measured amount of water to yield 3 to 10 γ of pyridone per ml. for measurement in the Beckman spectrophotometer, 2.5 ml. being required to fill the quartz cuvette. By utilizing the data of Knox and Grossman (2) readings are taken at 265 and 290 mμ. The absorption maximum at 265 mμ is much greater (× 2.5) than that at 290 mμ. On the other hand, many of the biologically important organic bases have high absorption values in the vicinity of 265 mμ and very low values near 290 mμ; this is particularly true of purines, pyrimidines, riboflavin, and pyridoxine, as well as of the other known derivatives of niacin. In view of this fact, it was gratifying to find that values obtained for the pyridone content of urines calculated from the readings at 265 and 290 mμ agreed remarkably well, as shown in Table II. This would indicate that the method of isolation employed in the procedure excludes fairly well the organic bases mentioned above normally present in urine.

TABLE II

Comparison of 6-Pyridone Values Measured with Different Fluorometers and Beckman Spectrophotometer

The values are recorded as mg. in a 24 hour urine.

Urine	Farrand	Coleman model 12A	Lumetron	Spectrophotometer	
				290 mμ	265 mμ
B.............	221	198	234	248	248
F.............	280	266	276	337	332
G.............	282	282	274	326	313

In Table II a few figures are shown comparing the results obtained on three 24 hour human urines, collected after the administration of 500 mg. oral doses of nicotinamide, when analyzed fluorometrically by using three types of fluorometer, and also spectrophotometrically. While the fluorometric results agree very well, the spectrophotometric figures are considerably higher. Since the absorption maxima of the free acid and the amide of the pyridone appear to be identical, whereas the fluorometric data include the amide only, the possibility of the presence of small amounts of the free acid should be considered. The other reasons for this discrepancy are not apparent and are being studied further. It should be noted, however, that attempts to obtain a satisfactory blank value for the spectrophotometric analysis were unsuccessful, and the values given in Table II were obtained by subtracting the absorption readings for similarly diluted and treated urines collected during the control periods from the same subjects. In the absence of a satisfactory method for determining the blank value, it is obvious that the spectrophotometric technique is not suitable for the analysis of normal control urines.

DISCUSSION

Preliminary studies showed that the proposed procedure involving treatment with lead and extraction with ether from a saturated K_2CO_3 filtrate, when applied to solutions of the pure (synthetic) pyridone in water or in "artificial urine," yielded extracts containing 90 to 100 per cent of the pyridone, as tested fluorometrically and spectrophotometrically. Solutions containing relatively large amounts of N^1-methylnicotinamide and the pyridone, when treated with lead and extracted with ether, gave the expected fluorescence values for the pyridone only, and tests employing the specific reaction for N^1-methylnicotinamide indicated its absence. This demonstrated the complete separation of N^1-methylnicotinamide and of its pyridone by the isolation procedure. It should be noted, however, that the acetone-KOH agitation reaction cannot be applied to a mixture of these two niacin derivatives, for under these conditions the N^1-methylnicotinamide yields a yellow compound which quenches the fluorescence of the pyridone compound, and on dilution with water yields the fluorescent condensation product described by Huff (6).

The choice of the alkali for the reaction with acetone was found to be restricted to KOH. Attempts to use NaOH, LiOH, and other strong metal and organic bases proved to be entirely ineffective. In the case of the metal bases this was shown to be due to the insufficient solubility of all but the KOH in anhydrous acetone. Even with the latter the solubility is so low that the yield of the fluorescent compound was very low and variable until the continuous shaking of the reaction mixture was adopted.

Any constant volume of 12 N KOH from 0.01 to 0.02 ml. for 0.7 to 1.0 ml. of acetone solution is satisfactory for this analysis, but extreme care must be taken to add the alkali accurately. The variation in the size of the individual drops, when delivered from a dropper or hypodermic needle attached to a syringe, made it necessary to use a micro pipette for adding the KOH. Good duplicate values and high fluorescence are obtained with 0.01 ml. of 12 N KOH. Larger amounts of 12 N KOH introduce more water into the reaction mixture and consequently there is a decrease in fluorescence. With smaller volumes of KOH, the reagent blank is considerably increased and the results erratic because of the inherent difficulty of measuring the smaller volumes of 12 N KOH with sufficient accuracy.

Because the intensity of fluorescence of the pyridone-acetone compound is very much less than that of the N^1-methylnicotinamide derivative, the fluorometric determination of small amounts of the former is correspondingly less accurate. Thus while the lower values in the range of 2.5 to 12 mg. of pyridone, found in a group of normal subjects, may not be closer than 15 to 30 per cent of the actual values, the higher ones, as shown in some examples of duplicate analyses quoted above, are within 5 to 10 per cent.

The necessity for the scrupulous exclusion of water other than that of the KOH solution during the reaction with acetone has been already emphasized. It is for this purpose that anhydrous Na_2SO_4 is used in the acetone reagent and in the preparation of the acetone solution for analysis.

The treatment with lead subacetate was introduced primarily for the clearing of normal control urines containing low amounts of the pyridone; it may possibly be omitted in the analysis of urines containing large amounts of pyridone.

Analyses of urines preserved with toluene and acidified to pH 3 to 4, which were kept in the refrigerator for as long as 2 months, showed that the pyridone content is unaffected.

The above procedure was applied successfully for the determination of the pyridone in human urines from normal subjects on ordinary diets and after oral doses of nicotinamide, and also in the urines of rats, dogs, rabbits, guinea pigs, and of Herbivora (7).

A trained analyst can complete the analyses of four urine samples by the fluorometric procedure in 1 day.

The chemical nature of the fluorescent product resulting from the reaction of the 6-pyridone of N^1-methylnicotinamide with acetone in the presence of alkali remains to be determined. The fact that this product is formed by the amide, but not by the free acid, suggests the possibility of a ring condensation product analogous to that described for N^1-methylnicotinamide (6). This aspect is being investigated.

SUMMARY

A fluorometric method for the determination of the 6-pyridone of N^1-methylnicotinamide in urine is described. It consists of clearing the urine with basic lead subacetate, extraction of the pyridone with ether from the lead filtrate saturated with K_2CO_3, evaporation of the ether and dissolving the residue in acetone, shaking of the acetone solution with a small amount of concentrated KOH, and fluorometric estimation of the resulting product. The pyridone can also be estimated spectrophotometrically in an aqueous solution of the residue after the evaporation of the ether extract.

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OROTIC ACID AS A PRECURSOR OF PYRIMIDINES IN THE RAT

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It was demonstrated by Richardson in 1936 (1) that uracil and guanine were essential for the growth of certain bacteria. Later several investigators found that purines and pyrimidines and corresponding nucleotides are of importance for the metabolism of different microorganisms. Snell and Mitchell (2) established the fact that guanine, adenine, uracil, and thymine may be limiting factors of growth for lactic acid bacteria.

In 1944 Loring and Pierce (3), working with pyrimidine-deficient mutants of *Neurospora*, found that orotic acid (uracil-4-carboxylic acid) and uracil had about the same growth-promoting effect, but that uridine and uridylic acid were 10 to 60 times more active. Simultaneously Rogers (4) reported that the growth of certain streptococci was stimulated to an equal degree by uracil and orotic acid, and Chattaway (5) observed that orotic acid was the only pyrimidine derivative that could replace the effect of liver extract on *Lactobacillus casei* ϵ.

These results indicated that orotic acid might be an intermediate in pyrimidine metabolism. Mitchell and Houlahan (6) proposed that oxalacetic, aminofumaric, and orotic acids may be intermediates in the synthesis of uridine and that the coupling with ribose occurred prior to the formation of the pyrimidine ring.

Orotic acid was discovered in milk in 1905 by Biscaro and Belloni (7). Recently it has been found to be produced in large amounts by certain *Neurospora* mutants. Genetic analysis of these strains led Mitchell, Houlahan, and Nyc (8) to the conclusion that orotic acid is not a normal intermediate in the biosynthesis of pyrimidines in *Neurospora* but is formed as a by-product in a side reaction. The occurrence in milk might be interpreted in a similar way.

In order to investigate this problem in animals, we have administered orotic acid containing N^{15} to rats.

EXPERIMENTAL

Orotic acid was synthesized according to Nyc and Mitchell (9) from aspartic acid containing about 12 atom per cent excess of N^{15}. The isotope is thus in the 3 position and the over-all excess N^{15} in the compound was found to be 6.06 per cent.

Two albino rats were each given 12.5 mg. of orotic acid per 100 gm. of body weight subcutaneously twice a day for 3 days. They were killed 12 hours after the last injection.

The polynucleotides were prepared from the pooled livers, pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) were fractionated (10), and the ribosides (11) from PNA and the purines from DNA (12) prepared and separated by partition chromatography on starch, according to the methods worked out earlier at one of our laboratories. The results are given in Table I.

The purines from DNA were not pure, according to the light absorption to nitrogen quotient.

The figures in the third column identifying each substance by means of maximum absorption and nitrogen content are in good agreement with the standard values. The prostate extract used for preparing the ribosides from the mononucleotides prior to the chromatographic separation was free from purines and pyrimidines, according to light absorption.

DISCUSSION

In work with pyrimidine-deficient *Neurospora* mutants cited earlier (3, 6, 13), it was proposed that in these organisms the free pyrimidines are not intermediates in the synthesis of polynucleotides, because free cytosine could not be utilized by uracil-deficient mutants, whereas cytidine or cytidylic acid supported growth. This has been taken to indicate that the interconversion of uracil and cytosine took place only when these pyrimidines were combined with ribose. Similar observations have been made by Fries (14) with pyrimidine-deficient strains of *Ophiostoma.*

The work by Plentl and Schoenheimer (15) has shown that rats did not utilize uracil or thymine for the synthesis of polynucleotides when the free pyrimidines were supplied in the diet.

The data in Table I show that part of the isotopic nitrogen in the injected orotic acid was recovered in uridine and cytidine with dilution factors of only 5.4 and 6.9 respectively. These low dilution factors prove that the administered orotic acid had entered into the metabolism as a precursor for the pyrimidine ring.

It is apparent from the isotope analysis that the orotic acid had not been utilized to any significant extent for the formation of purine ribosides, the purines in DNA, or of proteins.

The distribution of the isotope in the cytosine molecule between the ring and the amino group was not determined, since the amount of cytidine was insufficient for deamination.

TABLE I

Isolated substances	N^{15} atom per cent excess	$\frac{E_{\max.}*}{\gamma \text{ N per ml.}}$	Atom per cent N^{15}, calculated on basis of 100 per cent N^{15} in administered orotic acid
From PNA			
Adenosine	0.011	0.190	0.18
Guanosine	0.005	0.160	0.08
Cytidine	0.872	0.288	14.39
Uridine	1.133	0.314	18.70
From DNA			
Adenine	0.014		
Guanine	0.030		
Trichloroacetic acid-insoluble "protein"	0.009		0.15

* Determined on the following standards: adenosine at 257 mμ, 0.196; guanosine at 255 mμ, 0.164; cytidine at 280 mμ, 0.295; uridine at 262 mμ, 0.332.

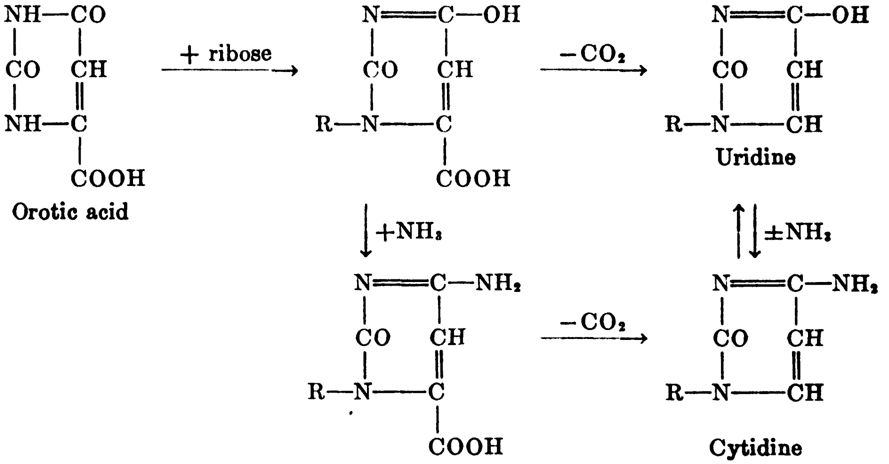

DIAGRAM 1. Possible reactions occurring in the utilization of orotic acid in biosynthesis of nucleosides.

The lower isotope content of cytidine (Table I) might be due to amination with non-isotopic nitrogen or with nitrogen of a low isotope content. Calculated on the assumption that we think is highly improbable, that all the excess isotope was contained in the ring, the value for the isotope excess in the cytosine ring becomes 1.308. On the assumption that the atoms per cent excess of N^{15} in the cytosine and uracil rings are equal, the excess in the amino group would be 0.350. On the other hand cytidine may not have been formed from uridine (see Diagram 1) and the observed difference might have significance, indicating different turnover rates of these pyrimidines.

The non-utilization of uracil, as demonstrated by Plentl and Schoenheimer (15), should indicate that corresponding coupling of the free pyrimidine does not take place in this animal. A possible explanation for the fact that orotic acid in contradistinction to uracil is utilized in the rat might be that a mechanism exists whereby orotic acid is coupled to ribose prior to decarboxylation (Diagram 1).

We hope to be able to answer some of these questions when work now in progress is completed.

In earlier experiments on rat liver some of us (16) found that glycine enters with some of its nitrogen (dilution 128-fold) into the pyrimidine ring. On the postulate that orotic acid is a normal intermediate in the biosynthesis of the pyrimidines, which of course is not shown by our experiment, glycine should be supposed to take part in this synthesis prior to the formation of orotic acid.

SUMMARY

Orotic acid containing N^{15} was administered to rats, and the nitrogenous compounds from liver nucleotides were tested for their content of isotope. The isotope was recovered in uridine and cytidine in 5.4- and 6.9-fold dilution of the administered orotic acid respectively, whereas the purine ribosides, the purine bases from DNA, and the proteins contained only insignificant amounts. It is concluded that the orotic acid had been utilized for synthesis of the pyrimidine ring, and different possibilities for the biosynthesis of the pyrimidine nucleosides are discussed.

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THE RÔLE OF INSULIN IN THE METABOLISM OF AMINO ACIDS

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It is well known that insulin exerts a profound influence on the metabolism of amino acids and protein. For example, the administration of insulin to the fasting, untreated diabetic has been shown to reduce the excessive excretion of nitrogen (1). Leutscher has recently observed that the diabetic has an unusually high concentration of blood amino acid and that insulin brings this to a normal value (2). Conversely, it has been demonstrated that the injection of insulin into the normal fasted man results in a depression of the concentration of blood amino acid to values below normal (3–5). This observation has been confirmed in the rat (3), the rabbit (3), and the dog (6). Harris and Harris (5) have studied the changes in the plasma levels of a limited number of individual amino acids in mental patients during insulin hypoglycemia. While the blood levels of all were depressed, leucine and lysine fell to the greatest extent. These authors offer no conclusive explanation for their findings.

That the liver is not specifically necessary for the action of insulin on amino acids has been shown by the fact that insulin delays the accumulation of blood non-protein nitrogen in the nephrectomized, eviscerated dog (7) and of amino acids in the blood of the eviscerated rat (8). Thus the effect of insulin on amino acids must be exerted on tissues in general, rather than in the liver or any other organ specifically.

In an attempt to understand more fully the nature of the rôle of insulin in amino acid metabolism, it was decided to study the effect of insulin on a representative number of individual amino acids and to examine the results in the light of the best evidence to date which indicates that insulin promotes the synthesis of protein (9, 7). Because of their particular importance in normal growth and nutrition, and because the methods for their analysis are most reliable, it was decided to study the ten "essential" amino acids.[1] The results of this study on the normal fasted dog are reported below, and support the belief that insulin is concerned with the synthesis of protein from free amino acids.

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* Scholar in the Medical Sciences of the John and Mary R. Markle Foundation.

[1] Leucine, lysine, arginine, isoleucine, valine, threonine, phenylalanine, methionine, histidine, tryptophan.

EXPERIMENTAL

Experiments were performed on two normal adult female mongrel dogs, maintained on a diet of laboratory chow, supplemented by horse meat twice a week. The animals were fasted for 18 to 24 hours before an experiment. After a control sample of blood was taken, insulin[2] was injected intravenously in a dose of 2 units per kilo of body weight. Blood samples, in amounts of 35 ml., were drawn into an oxalated syringe at 30 and 60 minutes thereafter. Neutralized tungstic acid filtrates of these bloods were prepared immediately and used for analysis of amino acids. The amino acid analyses were done by microbiological assay techniques. Each method was shown to give quantitative recovery of amino acid added to whole blood, and was therefore considered reliable for use. Only the natural forms of the amino acids are determined by these methods; therefore all data reported herein are for those forms only. Leucine, arginine, valine, threonine, methionine, histidine, and tryptophan were determined according to the method of Stokes *et al.* (10) with *Streptococcus faecalis* R as the test organism. Lysine and isoleucine were determined by a modification of the method of Dunn *et al.* (11) with *Leuconostoc mesenteroides* P-60 as the test organism. Phenylalanine was determined by a turbidimetric modification of the method of Henderson and Snell (12) with *Lactobacillus arabinosus*. Amino acid analyses of dog muscle were performed by these same methods on acid and alkaline hydrolysates of muscle. A biopsy of the thigh muscle was obtained under aseptic conditions. It was immediately minced and homogenized in a Waring blendor with distilled water. The homogenate was then put in a vacuum flask and lyophilized. The hydrolysates were made from the lyophilized muscle essentially according to the method of Stokes *et al.* (10).

Blood glucose was followed in all experiments as a check on the activity of insulin, the glucose being determined according to Nelson's photometric adaptation of Somogyi's method (13).

Results

It was found that after the intravenous administration of insulin the blood concentration of all ten amino acids fell, but to different degrees. It was further found that these differences remained characteristic from experiment to experiment. This observation lead to speculation concerning the reasons for these differences. If, as has been suggested (7), insulin plays a rôle in protein synthesis, then it would be logical to assume that under the influence of insulin each amino acid would be removed from the blood in quantities commensurate with the proportion of each in the protein in process of synthesis. It was therefore decided to test this hypothesis by comparing the proportions of each amino acid leaving the blood after insulin injection with the proportions of the same amino acids in a representative body protein. Skeletal muscle protein was chosen for this comparison, since it constitutes about 50 per cent of the body weight.

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[2] **Iletin, Lilly. The author is grateful to Eli Lilly and Company for the generous supply of insulin for use in this study.**

In preliminary experiments in which only three or four amino acids were measured at a time, it became apparent that such a correlation did exist and that the hypothesis was correct. In order to be sure, however, it was felt that it would be necessary to measure blood changes in all ten amino acids simultaneously. The techniques for microbiological assay were modified in order that all amino acids could be analyzed in the filtrate from 35 ml. of blood; the experiments were done on two dogs. In Table I are presented the data from such an experiment. Blood and muscle amino acid data were both obtained from the same dog. Since the blood leucine concentration was lowered to the greatest extent by insulin, and since leucine was in highest concentration in the muscle protein, leucine in both blood and muscle data was given a value of 10 and the other amino acids compared to it on the basis of relative molecular proportions. It is evident from the figures of Table I that in general there is a good correlation between the proportion of each amino acid in skeletal muscle protein and the proportion of each amino acid removed from the blood after insulin. This correlation is illustrated in graphic form in Fig. 1, in which the proportions of the amino acids removed from blood are plotted above the line, and the

TABLE I

Comparison between Relative Proportions of Ten Essential Amino Acids Removed from Blood after Insulin and Relative Proportions of Same Amino Acids in Dog Muscle Protein

Amino Acid	In blood				In muscle protein	
	Control	30 min.	Fall in concentration		Concentration, dry weight	
	µM per ml.	*µM per ml.*	*µM per ml.*	*molecular proportions*	*µM per ml.*	*molecular proportions*
Leucine	0.1740	0.0855	0.0885	10.0	0.5620	10.0
Lysine	0.4580	0.3840	0.0740	8.36	0.4910	8.74
Isoleucine	0.1167	0.0620	0.0547	6.19	0.4670	8.32
Arginine	0.2370	0.1860	0.0510	5.77	0.3080	5.48
Valine	0.1400	0.0962	0.0438	4.95	0.4080	7.26
Threonine	0.1665	0.1227	0.0438	4.95	0.3700	6.58
Phenylalanine	0.0582	0.0327	0.0255	2.88	0.2210	3.93
Methionine	0.0537	0.0295	0.0242	2.74	0.1530	2.72
Histidine	0.0806	0.0626	0.0180	2.04	0.1500	2.67
Tryptophan	0.0304	0.0227	0.0077	0.87	0.0223	0.397

muscle amino acids below it. These data, then, substantiate the hypothesis and show that under the influence of insulin the ten essential amino acids do indeed leave the blood in quantities proportional to their concentrations in a representative body protein.

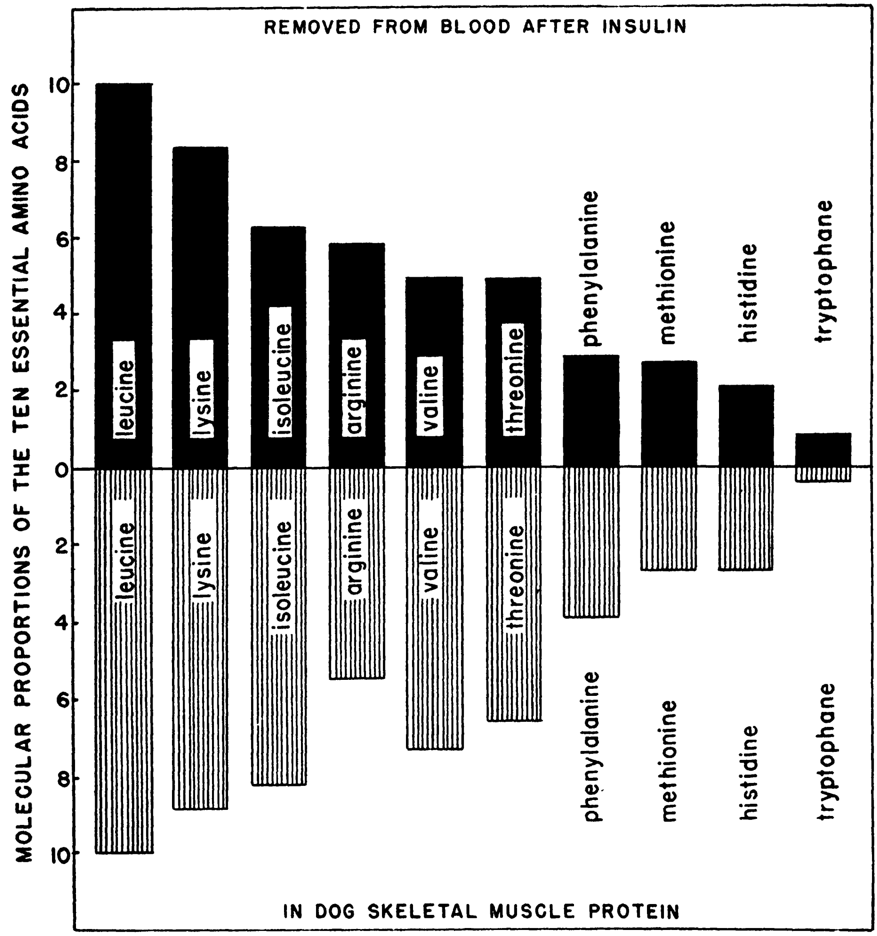

FIG. 1. A comparison between the molecular proportions of the ten essential amino acids removed from the blood of a dog after insulin and the proportions of the ten essential amino acids in the same dog's skeletal muscle protein.

DISCUSSION

These data suggest three possible explanations for the action of insulin on amino acids. First, insulin might promote increased deamination. This is unlikely in view of the fact that insulin affects amino acids even in the absence of the main deaminative organ, the liver. Furthermore, the data of Bach and Holmes (14) and Stadie *et al.* (15) indicate that insulin actually inhibits, rather than accelerates, the oxidative deamination of amino acids by liver slices.

Second, insulin might possibly depress the hydrolysis of protein and thus bring about a decrease in the quantity of amino acid entering the blood. This possibility cannot be absolutely ruled out on the basis of the experiments reported here. However, if insulin simultaneously inhibits both the hydrolysis of tissue protein and deamination of amino acids, it would seem highly improbable that the proportionality pattern observed here would have occurred.

Third, as has been suggested, insulin might play a rôle in the process of protein synthesis. This interpretation would explain the data adequately and furthernore would be in harmony with other evidence implicating insulin in protein synthesis. Grey and Thalhimer (16) in 1924 were able to demonstrate greater growth of chick fibroblasts in tissue culture with insulin and glucose than with glucose alone. Mirsky (17) has shown that anterior pituitary extract requires insulin for its protein synthetic function. Indeed in the diabetic animal this extract actually promotes the breakdown of tissue protein rather than its synthesis. This interrelation between insulin and the pituitary was also shown by Frame and Russell (8) who observed that insulin and anterior pituitary extract together are more effective than insulin alone in decreasing the rate of accumulation of amino acids in the blood of the eviscerated rat.

Wilhelmi, Fishman, and Russell (18) have recently stated that "the activity of the anterior pituitary gland in maintaining normal levels of muscle glycogen in the 24 hour fasted hypophysectomized rat . . . appears to be a property of the growth hormone." Thus insulin is not only involved in the protein anabolic function of the pituitary, but the pituitary is also apparently involved in glycostasis, a function known to be affected by insulin.

These studies, linking insulin and the pituitary in both glycostasis and protein synthesis, emphasize the interrelated nature of their functions and add weight to the feeling that the data presented in this paper are most logically interpreted in the light of a protein anabolic function of insulin. Experiments are now planned in which the effects of insulin and growth hormone on the metabolism of the individual amino acids will be studied *in vivo*, and with muscle strips *in vitro*. It is hoped that these studies will add further meaning to the experiments reported in this paper.

SUMMARY

The effect of insulin on the blood concentration of the ten essential amino acids has been studied in the normal fasted dog. It has been shown that there is a correlation between the proportions of each amino acid re-

moved from blood after insulin and the proportions of each amino acid in a representative body protein, skeletal muscle. It has been suggested on the basis of this observation that insulin promotes the synthesis of protein from circulating amino acids. This interpretation of the data is in keeping with recent evidence that points to an intimate relationship between insulin and the anterior pituitary gland in both protein and carbohydrate metabolism.

The author wishes to express his thanks to Dr. Robert Pitts and Dr. Jay Tepperman for their helpful suggestions and to Mr. Raymond Cottet for his assistance in the performance of the experiments.

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THE BIOLOGICAL ACTIVITY OF SUBSTITUTED PURINES*

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It has been shown that the animal microorganism, *Tetrahymena geleii*, requires an exogenous source of purine for growth (1). This requirement can be met by the addition of guanine (or guanosine or guanylic acid) to the medium (2). No other naturally occurring purine can replace guanine, although adenine and hypoxanthine exhibit marked sparing action. It was pointed out that *Tetrahymena* differs from mammals in that it is capable of synthesizing adenine from guanine but not guanine from adenine, whereas in the mammal the reverse appears to be true (3).

A systematic testing of substituted pyrimidines has been shown to be a means of evaluating the active positions and radicals (4), and accordingly a similar study has been made with twenty-one substituted purines. With these compounds, as with the pyrimidines, conclusions were drawn on the basis of replacement, sparing action, inhibitory action, and inactivity.

The compounds tested were generously supplied to us by Dr. George H. Hitchings and his colleagues of the Wellcome Research Laboratories, to whom we are deeply grateful.

Methods

The methods employed are identical with those used in the investigation of the substituted pyrimidines (4). The basal medium differed in one respect only. Guanylic acid and adenine were omitted and uracil and cytidylic acid were added (10 γ per ml. and 25 γ per ml. respectively). Tests for replacement, sparing action, and inhibition were conducted in the manner previously described (4), with similar ranges being tested.

Results

Table I summarizes the results obtained. Of the substituted guanines available for testing, two were capable of supplying the purine requirement (replacement of guanine) and two exhibited weak guanine sparing action. Substitution of position 1 with a —CH_3 group reduced the activity for replacement of guanine (rated as 100 per cent) to 75 per cent. In this case the growth produced by 8 γ per ml. of 1-methylguanine equaled that

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* Aided by a grant from the United States Public Health Service and a grant recommended by the Committee on Growth of the National Research Council, acting for the American Cancer Society.

produced by 6 γ per ml. of guanine hydrochloride. On the other hand 8-methylguanine, while it will replace guanine, is very difficultly metabolized by the organism. Activity is about 4 per cent and optimum growth is not obtained even at high levels. The growth rate is markedly reduced.

Substitution in position 7 (7-methylguanine) destroys replacement activity, but slight sparing action occurs after prolonged growth periods. Added substitutions in position 1 (1,7-dimethylguanine) reduce activity further. It appears that, in the presence of small amounts of guanine, these two compounds can be demethylated at a very slow rate.

TABLE I

Activity of Substituted Purines

Replacement	Per cent activity*	Sparing	Inhibition	Inhibition index†	Inert
1-Methylguanine	75	Adenine	5-Amino-7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine	0.075	7-Hydroxy-1*H*-*v*-triazolo[*d*]-pyrimidine
1-Methylxanthine	15	Hypoxanthine	5,7-Diamino-1*H*-*v*-triazolo[*d*]-pyrimidine	85	Xanthine
2,6-Diaminopurine	2.3	7-Methylguanine (poor)	1,3,7-Trimethylxanthine (caffeine)	100‡	7-Methylxanthine
8-Methylguanine	4 (slow)	1,7-Dimethylguanine (poor)	3,7-Dimethylxanthine (theobromine)	150‡	3-Methylxanthine
			1,3-Dimethylxanthine (theophylline)	225‡	2-Thio-6-ketopurine
			1,7-Dimethylxanthine (paraxanthine)	300‡	Uric acid

* Calculated from guanine, which was rated 100 per cent.

† The inhibition index is here defined as the smallest ratio between the amount of the inhibitor and the antagonist at which growth does not occur.

‡ These indices were determined by using low concentrations of guanylic acid. Raising the purine concentration of the medium changes the index.

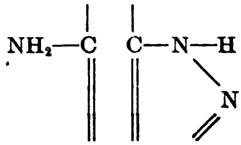
The activity of adenine and hypoxanthine as sparing agents was reported earlier (2). Neither of these compounds can replace guanine. If, however, position 2 of adenine is aminated (2,6-diaminopurine), then the organism is able to use this compound as a substitute for guanine. The activity is low (2.3 per cent), however, and optimum growth is never attained.

Xanthine is without activity (2) but 1-methylxanthine can replace guanine. This was an unexpected finding and the explanation is not immediately at hand. The activity is about 15 per cent, which is far too high to be accounted for on the basis of guanine contamination. It may be that the methylation of position 1 "fixes" the keto radical at position 6 (no enolization). It is then possible for the organism to aminate the 2 position to produce 1-methylguanine.

All other methylxanthines tested were either inhibitory (caffeine, theobromine, theophylline, paraxanthine) or inert (7-methylxanthine, 3-methylxanthine). Aside from the single methyl substitution in position 1 mentioned above, there tends to be an inverse relationship between the number of —CH_3 substitutions and the activity of the compound. Thus 7-methylxanthine and 3-methylxanthine are inert. When both these positions are filled with —CH_3 groups (theobromine), an inhibitory compound is produced. Substitution of position 3 or 7, together with position 1 (theophylline and paraxanthine), produces inhibitors, and even stronger inhibition results when all three positions (1, 3, and 7) are —CH_3-substituted (caffeine).

It has so far been impossible to release more than about half of the inhibitory effect of the methylxanthines with guanine or any other purine or mixtures of purines. Best results in antagonizing the inhibition have been obtained with a mixture of guanine, xanthine, and adenine. The methylxanthines appear to inhibit more than one enzyme system. The non-purine systems affected seem to have a higher resistance than the purine systems. Thus, at relatively low levels of inhibitors, complete release can be obtained with the purine mixture but, at higher levels of inhibitors, no further release results. Raising the levels of the B vitamins as much as 100-fold or the addition of nicotine (5) had no effect. The nature of the non-purine system or systems interfered with by the methylxanthines must await further investigation.

The triazolo compounds were first synthesized by Roblin *et al.* (6). The structural relationships with the purines can be seen if representation is made as follows:



5-Amino-7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine

In this form positions 5 and 7 of the triazolo compounds become the equivalent of positions 2 and 6 of the purines. In effect, therefore, the 5-

amino-7-hydroxytriazolo compound can be thought of as guanine (enol form) with a nitrogen replacing the 8 position carbon.

This triazolo compound is by far the most powerful inhibitor encountered in this investigation. The inhibition index is 0.075. That is to say that 1 molecule inhibits 13 to 14 molecules of guanine. This inhibition can be completely reversed by guanine. Roblin *et al.* (6) found this compound to be inhibitory to certain bacteria, the inhibition being specifically antagonized by guanine. The inhibition ratios were much higher, however, than is shown with *Tetrahymena.*

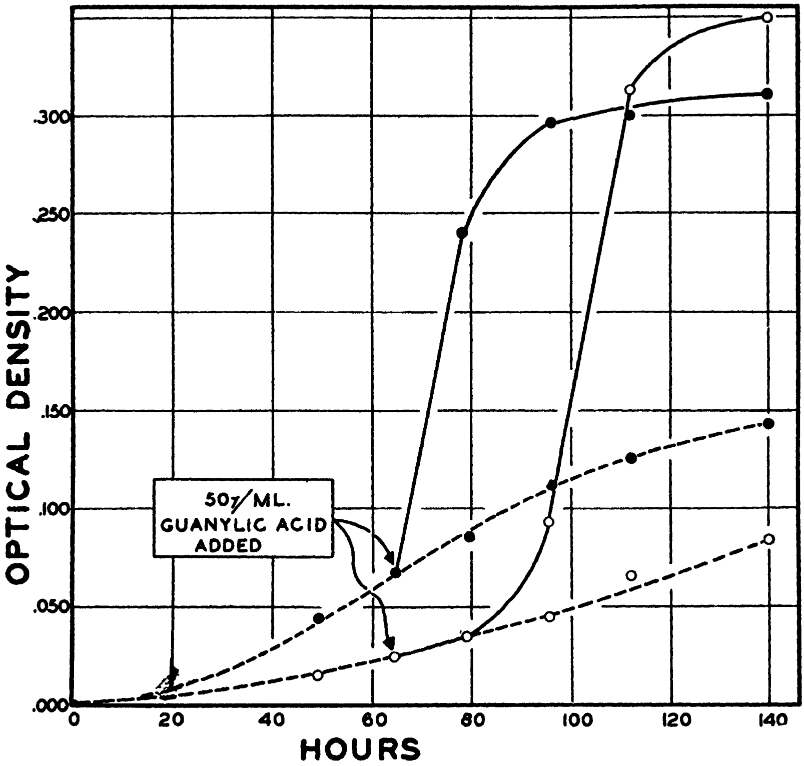


FIG. 1. Growth curves for two purine inhibitors and the effect of added guanylic acid. ○, growth when 0.4 γ per ml. of 5-amino-7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine was present with an initial level of 12 γ per ml. of guanylic acid. ●, growth when 60 γ per ml. of caffeine were present with an initial level of 5 γ per ml. of guanylic acid. The solid lines represent the growth of the organisms after addition of guanylic acid, while the dash lines represent the growth without added guanylic acid. Caffeine-inhibited organisms respond immediately to the addition of antagonist (guanylic acid), while the 5-amino-7-hydroxytriazolo-inhibited organisms do not respond appreciably for many hours.

Tests were conducted wherein graded amounts of 5-amino-7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine were added to the basal medium together with suboptimum amounts (12 γ per ml.) of guanylic acid, and growth was followed by readings at regular intervals. For comparison, a series with caffeine (with guanylic acid at 5 γ per ml.) and two pyrimidine inhibitors (isobarbituric acid and 2-thiouracil) was similarly followed. In the last two cases suboptimum amounts of uracil (4) were used. Optimum amounts of antagonists (guanylic acid or uracil) were added aseptically after 64 hours of incubation. The control series, receiving no antagonist, was followed for the 140 hours of the duration of the experiment. Growth rates were proportional, in all cases, to the amount of inhibitor present during the first 64 hours of incubation, and throughout the experiment in the controls. Immediate response in increased growth rate occurred when the antagonist was added to the caffeine, isobarbituric acid, and 2-thiouracil series. In the case of the 5-amino-7-hydroxytriazolo compound, however, the establishment of the optimum growth rate was delayed 20 to 30 hours after addition of the antagonist. Growth curves for single levels of each of two inhibitors are shown in Fig. 1.

When the 7 position (6 of the purine) is filled by an amino group (5,7-diamino-1*H*-*v*-triazolo[*d*]pyrimidine), the inhibition index is 85. Again guanine is capable of complete release. This triazolo compound is therefore about as effective a guanine inhibitor as 2,6-diaminopurine, which it closely resembles from a structural standpoint, is a replacer of guanine.

When the 5 position is unsubstituted (7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine), the compound is inert.

DISCUSSION

Competitive inhibition is thought to result when a compound is similar enough structurally to a required metabolite to fit the enzyme system responsible for the metabolism of the metabolite. The metabolic block would be due to the inability of the organism to make use of the inhibitor. If this were true, therefore, the most powerful inhibitors would be those with structures most nearly resembling those of required compounds.

The extreme inhibitory activity of 5-amino-7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine may be due to its complete primary utilization by the guanine-enzyme system, to the extent that its nucleoside (and probably nucleotide) is formed in place of those of guanine. If this abnormal nucleotide is formed, however, it would appear to be fatal to the organism during later stages of development. This situation would be overcome only if enough guanine were present so that the formation of the abnormal nucleotide would be negligible. Evidence supporting this hypothesis is seen in Fig. 1. Relatively mild inhibitors, which may compete with the antagonist at the enzyme surface, are immediately overcome by large amounts of antagonist, while appreciable time is required for overcoming the effects of the triazolo compound. These results indicate to us a more intimate metabolic association on the part of the latter compound than milder inhibitors show.

The inhibitory activity of 5-7-diamino-1*H*-*v*-triazolo[*d*]pyrimidine is of such an order as to suggest competition with guanine at the primary enzyme surface with no further metabolism of the compound.

It was earlier suggested (4) that the testing of analogues of the pyrimidines with the animal microorganism *Tetrahymena* might be a useful means of screening compounds for bacteriostatic action and therapeutic usefulness. Those compounds having low inhibition indices for *Tetrahymena* could be excluded from consideration as useful agents.

The present investigation throws a somewhat different light on these considerations, due to a peculiarity of *Tetrahymena* when compared to the mammal. *Tetrahymena* is incapable of synthesizing guanine from adenine (2), while the reverse seems to be the case in mammals (3). If such organisms as blood flagellates (*Trypanosoma*), tissue flagellates (*Schizotrypanum, Leishmania*) intestinal amoebae (*Endamoeba histolytica*), malarial parasites, viruses, and rickettsiae should be found to follow the biochemical pattern of *Tetrahymena* and require guanine, then they should be powerfully inhibited by the 5-amino-7-hydroxytriazolo compound, which in turn should possess little or no inhibitory action toward the mammalian host.[1] This compound deserves serious consideration as a likely therapeutic agent.

SUMMARY

Twenty-one substituted purines were tested for replacement and sparing of guanine and inhibitory action, with the animal microorganism *Tetrahymena geleii*.

Four (1-methylguanine, 8-methylguanine, 1-methylxanthine, and 2,6-diaminopurine) were found capable of supplying the purine requirement, replacing guanine. Four (adenine, hypoxanthine, 7-methylguanine, 1,7-dimethylguanine) can spare guanine but not replace it.

The triazolo analogue of guanine (5-amino-7-hydroxy-1*H*-*v*-triazolo[*d*]-pyrimidine) is a powerful purine inhibitor with an inhibition index of 0.075. The inhibition is completely reversed by guanine. Of two other triazolo

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[1] In a series of preliminary tests, mice were given intravenous, subcutaneous, and intraperitoneal injections of 5-amino-7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine. No apparent toxicity resulted in any case, even though the compound was given at levels of 100 γ per gm. of mouse weight and this dose repeated three times daily for 3 days. The compound did not affect the fatal course of infection of mice with *Trypanosoma rhodesiense*, however. This might indicate the non-identity of the purine pattern of metabolism of the flagellate with that of *Tetrahymena*, which would not be unexpected, considering the generally accepted plant origin of the flagellate stock.

compounds tested, one was moderately inhibitory (5-7-diamino-1*H*-*v*-triazolo[*d*]pyrimidine) and the other (7-hydroxy-1*H*-*v*-triazolo[*d*]pyrimidine) was inert. The dimethylxanthines (theobromine, theophylline, paraxanthine, and trimethylxanthine (caffeine) were found to be inhibitory, but the inhibition was only partly reversed by guanine or other purines. The residual inhibition was not reversed by known vitamins or nicotine.

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INHIBITION OF THE PROTEINASE AND ESTERASE ACTIVITIES OF TRYPSIN AND CHYMOTRYPSIN BY DIISOPROPYL FLUOROPHOSPHATE: CRYSTALLIZATION OF INHIBITED CHYMOTRYPSIN*

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The discovery of the esterase activity of trypsin and chymotrypsin (1, 2) has naturally raised the question whether the two activities are referable to the same or to different functional groups in these proteins. Schwert *et al.* (1) have concluded that the hydrolysis of α-benzoyl-L-arginine methyl ester and α-toluenesulfonyl-L-arginine methyl ester was catalyzed by trypsin itself and by the same active surfaces responsible for the proteolytic activity (as measured by the hydrolysis of α-benzoyl-L-arginineamide) on the basis of the following evidence: (*a*) All crystalline trypsin preparations had approximately the same esterase activity regardless of the method of purification, (*b*) the autolysis of trypsin in alkaline solution resulted in a parallel decrease in both activities, (*c*) the pH optimum for casein and ester hydrolysis was the same, and (*d*) the addition of increasing amounts of crystalline soy bean inhibitor progressively reduced both activities.

It occurred to us that the highly selective inhibition of certain esterases by various organic phosphates, notably diisopropyl fluorophosphate (DFP) and tetraethyl pyrophosphate (TEP), might furnish additional evidence for the similarity or dissimilarity of the centers of proteinase and esterase activities. It has been shown that both true and pseudo cholinesterases are readily inhibited by DFP (3) and by the mixture called hexaethyl tetraphosphate of which TEP is the active constituent (4). Furthermore the inhibitory action of DFP has been regarded as strictly confined to certain esterases (5),[1] and a preliminary test showed us that the esterase activity of both trypsin and chymostrypsin was in reality strongly inhibited by DFP. Accordingly, a test was made to see whether both activities were similarly affected. The work presented here shows that this is the case, and thus supports the view that a single group is responsible for both enzyme actions.

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* Enzyme Research Division Contribution No. 118.

[1] The inhibition is generally considered irreversible *in vitro*, although this may depend on factors other than the enzyme itself, for in the case of (citrus) acetylesterase we have observed TEP inhibition to be reversible *in vitro* and DFP inhibition to be reversible in the intact fruit (6).

Chymotrypsinogen, which is neither an esterase nor a proteinase *per se*, was not affected in any observed fashion by a concentration of DFP 10 times that needed to cause complete inactivation of the corresponding quantity of chymotrypsin. After treatment it could be recrystallized in typical form and activated with trypsin to give chymotrypsin with the same proteinase and esterase activity as chymotrypsin resulting from untreated chymotrypsinogen.

On the other hand, both the proteinase and esterase activities of crystalline trypsin and crystalline chymotrypsin were readily inhibited by small concentrations of DFP (though not by relatively large concentrations of TEP). With each enzyme the inhibition of both activities occurred at essentially the same rate and to the same extent.

The foregoing results agree with the postulate that one group is responsible for both the esterase and proteinase activities in trypsin and chymotrypsin. The same conclusion has been reached by an entirely different method, whereby trypsin was partially inactivated by hydrostatic pressure. In this case also the loss of esterase activity paralleled that of proteinase activity.[2] However, the existence of two kinds of active groups in trypsin is a distinct possibility from the observation of Fraenkel-Conrat, Bean, and Lineweaver (7) that acetylated trypsin, although still proteolytic, can no longer be readily inhibited by ovomucoid, the antitrypsin of egg white. A sample of acetyl trypsin[3] showed only about half as much proteinase activity per measure of esterase activity as found for crystalline trypsin. It is true that when treated with DFP this sample lost both proteinase and esterase activity, but it required much more inhibitor, and the loss of proteinase activity was disproportionally greater than that of esterase activity. This observation is in direct contrast to all the others reported here and will be discussed later.

Extremely small quantities of DFP were found to be sufficient for the inhibition of chymotrypsin. In equimolar proportions the enzyme was almost totally inhibited.[4] The chance of obtaining DFP-inhibited chymotrypsin in crystalline form seemed therefore to be good, and the attempt was successful. Crystalline chymotrypsin was completely inhibited by DFP and the resulting product recovered in crystalline form. After one recrystallization, the resulting protein showed no esterase or proteinase ac-

[2] Part of an investigation of the effect of high pressures on enzymes, sponsored by Bankhead-Jones funds. The results will be published shortly.

[3] Kindly furnished by Dr. H. Fraenkel-Conrat.

[4] Trypsin required more inhibitor possibly because the pH for the optimal effect of DFP was not used on account of the instability of trypsin in that range.

tivity whatever. Furthermore it exerted no inhibitory effect on active chymotrypsin, so presumably it contained no free DFP. The crystals were (to us) indistinguishable from ordinary α-chymotrypsin. This substance appears to be one of the few known examples of an artificially inhibited enzyme in the crystalline state. Kunitz and Northrop (8, 9) have prepared crystalline trypsin-inhibitor complexes and Herriott and Northrop (10) crystallized acetylated derivatives of pepsin whose activities varied with the degree of acetylation. We believe that DFP-inhibited chymotrypsin will also serve as a useful material for studying the mode of action of some hydrolytic enzymes, as well as the specific effect of DFP thereon. The results of further study on this crystalline product will be reported later.

Materials and Methods

Inhibitors—The DFP[5] was analytically pure, the TEP[6] was at least 95 per cent pure, and the diethyl *p*-nitrophenyl thiophosphate (parathion)[7] was technical grade. Stock solutions of the inhibitors were made to be 0.1 to 0.001 M in anhydrous isopropanol; such solutions were stable for several months in the refrigerator. Aliquots of these solutions sufficient to give the desired concentration of inhibitor were added to an enzyme solution and incubated at room temperature (25° ± 1°) for a specified time previous to assay. As a control, pure isopropanol was added in a corresponding amount to a similar enzyme solution in order to ascertain whether any of the inhibition observed might be due to the isopropanol. In no case did the amount of isopropanol used produce any inhibition over the time of the experiment.

Enzymes and Assay Methods—The crystalline chymotrypsinogen[8] was recrystallized seven times after receipt, then exhaustively dialyzed and lyophilized. The crystalline chymotrypsin[9] and trypsin[10] were obtained as dry filter cakes. The proteinase activities of trypsin and chymotrypsin were determined by the Anson hemoglobin method (11). The esterase activity of trypsin was determined by a continuous potentiometric titration procedure similar to that used by Schwert *et al.* (1), in which the carboxyl groups liberated by the hydrolysis of α-toluenesulfonyl-L-arginine methyl ester (TSAME) were neutralized with 0.02 N NaOH. No buffer was needed with this dilute alkali. The 20 ml. reaction mixture consisted of sufficient TSAME to make it 0.01 M and, routinely, an amount of enzyme which would cause the liberation of 0.005 m.eq. of carboxyl groups per minute. The pH was maintained at 7.5 by the addition of 0.02 N NaOH, and periodically readings were made of the alkali added. The hydrolysis (with trypsin) proceeded as a zero order reaction. The results are expressed in milliequivalents of carboxyl groups liberated per minute per mg. of protein nitrogen, or in the per cent of a control. The esterase activity of chymotrypsin was likewise determined by a continuous titration procedure, L-tyrosine ethyl ester (TEE) being used as a substrate. TEE was hydrolyzed at the same rate as benzoyl-L-tyrosine ethyl ester, but TEE had the advantage of being more soluble in water. The conditions of assay were the same as those used for trypsin; *i.e.*, the substrate concentration was 0.025 M, and the pH of the assay was 7.5. Since the reaction with chymotrypsin was found to be one of first order, the results were calculated in terms of milliequivalents of carboxyl groups liberated per minute on the basis of the initial slope.

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[5] Obtained from the Medical Division, Army Chemical Center, Edgewood, Maryland, through the courtesy of Captain James A. Campbell.

[6] Kindly supplied by Dr. Howard Adler of the Victor Chemical Works, Chicago.

[7] Kindly supplied by Mr. A. F. Kirkpatrick of the American Cyanamid Company, New York.

[8] Purchased from the Plaut Research Laboratory, Lehn and Fink Products Corporation, Bloomfield, New Jersey.

[9] Purchased from the Armour Laboratories, Armour and Company, Chicago.

[10] Kindly supplied by Dr. M. Kunitz of the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey.

The symbols $[\text{Enz. u.}]^{\text{substrate}}_{\text{mg. or mg. PN}}$ established by Northrop and his group were used to express activities; *e.g.*, $[\text{T. u.}]^{\text{TSAME}}_{\text{mg. PN}}$ represents the trypsin units with the use of TSAME as a substrate (milliequivalents of COOH groups liberated per minute) per mg. of protein nitrogen.

The term pK is used to denote the negative logarithm of the concentration of inhibitor required to produce 50 per cent inhibition.

EXPERIMENTAL

Failure of DFP Treatment to Alter Chymotrypsinogen—Schwert *et al.* (1) found that chymotrypsinogen failed to hydrolyze α-benzoyl-L-arginine methyl ester, whereas chymotrypsin hydrolyzed this substrate well. Similar results were obtained by us with TEE, with which 15 mg. of chymotrypsinogen failed to cause any detectable hydrolysis. Hence the conversion of the zymogen to the active enzyme uncovers both the proteinase and esterase centers of activity.

Chymotrypsinogen was dissolved in 0.2 M phosphate buffer of pH 7.7 to give a concentration of 15 mg. per ml. Sufficient DFP was added to give a final concentration of 0.001 M. After 24 hours 1 ml. of the solution was diluted with 6 ml. of buffer, and 0.01 mg. of trypsin in 2 ml. of 0.005 N HCl was added. After incubation at 5° for 24 hours the activity[11] was $[\text{Xt. u.}]^{\text{TEE}}_{\text{mg.}} = 0.0028$. (A control sample of untreated chymotrypsinogen which was converted under the same conditions gave an identical activity.) The chymotrypsin was incubated with 0.00005 M DFP at 25° for 20 minutes, after which no TEE activity was detectable.

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[11] The activities are expressed on the basis of the weight of the chymotrypsinogen used to convert to the active enzyme.

In another experiment 2.4 gm. of chymotrypsinogen were dissolved in 160 ml. of 0.2 M phosphate buffer at pH 7.7 and then treated with sufficient DFP to give a final concentration of 0.001 M. After 24 hours the pH was adjusted to 5.0 with 2 M H_2SO_4, and 78 ml. of saturated ammonium sulfate were added. On standing at 5° for 24 hours, typical chymotrypsinogen crystals formed. A further crop of crystalline chymotrypsinogen was obtained by precipitating the residual protein in the mother liquor by 0.9 saturation with ammonium sulfate and treating this precipitate according to the method of Kunitz and Northrop (12). The crystalline material was suspended in water, exhaustively dialyzed against water, and lyophilized. The yield was 870 mg. or 36 per cent. On conversion to chymotrypsin with trypsin under conditions similar to the above, but for 48 hours (longer incubation failed to cause an increase in activity), the activities[11] were $[\text{Xt. u.}]^{\text{Hb}}_{\text{mg.}} = 0.0047$ and $[\text{Xt. u.}]^{\text{TEE}}_{\text{mg.}} = 0.0049$. A control sample of untreated chymotrypsinogen gave 10 per cent less esterase activity but an identical proteinase activity. On treatment with DFP to give a concentration of 0.00005 M and with a concentration of 1 mg. of enzyme per ml., both the chymotrypsin of the control and also that derived from the DFP-treated chymotrypsinogen were completely inhibited. Hence, if there was a reaction between DFP and chymotrypsinogen, it did not affect the conversion to active chymotrypsin, or the inhibition of the resulting enzyme by DFP.

Effect of DFP on Trypsin and Acetylated Trypsin—Contrary to the results observed with acetylesterase (6), the pH of the reaction contributed greatly to the inhibition of trypsin by DFP. When trypsin (0.025 mg. per ml.) was incubated in 0.0001 M DFP at pH 3.5, 4.5, 5.0, and 6.0 for 20 minutes prior to assay, it was found that 0, 9, 23, and 67 per cent inhibition had occurred. However, because of the well known instability of trypsin at pH values close to neutrality, a compromise at pH 5 was used for the results reported here. For this reason the results on trypsin are not quantitatively comparable to those obtained with chymotrypsin, as in the latter case it was possible to use a pH at which the reaction with DFP proceeded rapidly, yet at which the pH was without effect on the chymotrypsin.

In Fig. 1 typical results are presented of the inhibition of trypsin and acetylated trypsin with DFP. The inhibition reactions proceeded as bimolecular reactions similar to that observed for the inhibition of acetylesterase (6). Incubation of trypsin in the presence of 4×10^{-4} M DFP was sufficient to cause well over 80 per cent inhibition in 24 hours. On the other hand, acetylated trypsin was inhibited at a much slower rate with a given concentration of DFP. At a DFP concentration of 4×10^{-4} M the inhibition did not go over 50 per cent and was incomplete even at 10×10^{-4} M DFP. Furthermore, the inhibitions of the two activities were not parallel as in the case of unchanged trypsin. The reason for these differences is not apparent. However, the sample of acetylated trypsin (7), in which essentially only amino groups were acetylated (amino N had been decreased from 1.2 to 0.4 per cent), and which had been stored in the cold for several months, had the activities $[\text{T. u.}]^{\text{Hb}}_{\text{mg. PN}} = 0.085$ and $[\text{T. u.}]^{\text{TSAME}}_{\text{mg. PN}} = 1.59$. The ratio of TSAME:Hb was 18.7. The activities of trypsin are $[\text{T. u.}]^{\text{TSAME}}_{\text{mg. PN}} = 1.75$ and $[\text{T. u.}]^{\text{Hb}}_{\text{mg. PN}} = 0.17$, giving a ratio of 10.3.

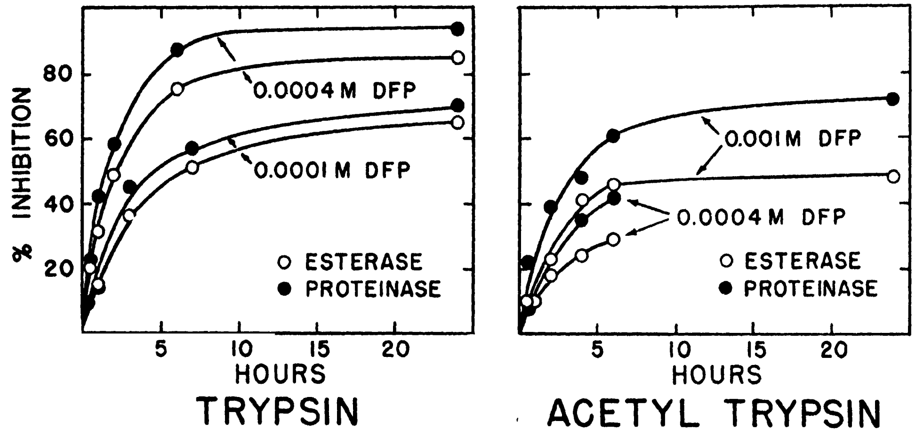

FIG. 1. The inhibition of the esterase and proteinase activities of trypsin and acetyl trypsin by DFP. The concentration of trypsin was 0.004 mg. of protein nitrogen per ml. in 0.2 M acetate buffer at pH 5.0. The concentration of acetyl trypsin was 0.003 mg. of protein nitrogen per ml. in the same buffer.

In conjunction with Fraenkel-Conrat, the effect of acetylation on the activities of trypsin was studied further. It was found in two additional experiments that freshly prepared acetyl trypsin (acetylated to the same extent as the above) had an esterase activity identical to that of untreated trypsin but a proteinase activity of approximately 50 per cent. The acetyl trypsin was less prone to autolysis in solution than is trypsin. The activity-substrate-concentration relationship was the same for the hydrolysis of TSAME or hemoglobin by trypsin and acetyl trypsin. Hence the decrease in the proteinase activity of acetyl trypsin was real and not due to a difference in the affinity of enzyme for the substrates.

Effect of DFP on Chymotrypsin—The effect of pH on the inhibition of chymotrypsin by DFP was similar to that observed with trypsin. The most effective pH for the inhibition reaction was 7.5. Since chymotrypsin is stable at this pH, all the experiments with chymotrypsin were carried out at pH 7.5 to 7.7.

In Fig. 2 the results obtained on the inhibition of α-chymotrypsin by DFP are presented. A 20 minute exposure period of enzyme to DFP was used prior to assay. Unlike acetylesterase (6), and trypsin (Fig. 1), the reaction of DFP with chymotrypsin was finished in less than 5 minutes. At the low concentrations of DFP used in this experiment, longer incubation periods failed to cause any further inhibition. The esterase and proteinase activities of the chymotrypsin were essentially equally inhibited at any given concentration of DFP (Fig. 2). Hence both activities of chymotrypsin appear to involve the same active centers. The point of 50 per cent inhibition was found to be 8 × 10^{-6} M DFP. When the molar ratio of DFP to chymotrypsin (with use of 27,000 for the molecular weight of chymotrypsin,[12] which has a nitrogen content of 15.83 per cent (13)) was 1:1, practically total inhibition occurred. This is indeed a small amount of inhibitor, and indicates that there are not more than one or possibly two active centers in the chymotrypsin.

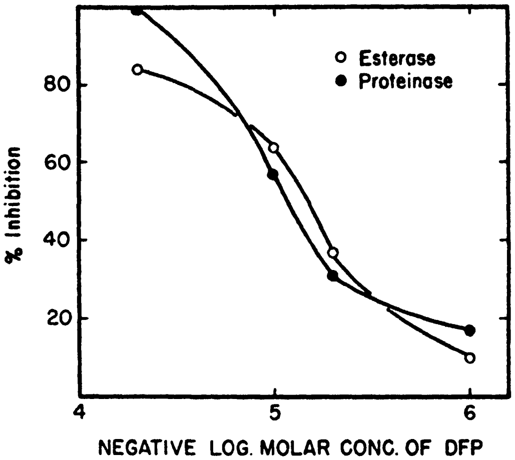

FIG. 2. The inhibition of the esterase and proteinase activities of α-chymotrypsin by DFP. The concentration of α-chymotrypsin was 0.060 mg. of protein nitrogen per ml. in 0.2 M phosphate buffer at pH 7.7; the time of exposure was 20 minutes at room temperature.

A similar experiment with β-chymotrypsin gave identical results. The curves obtained with an equal quantity of β-chymotrypsin were practically superimposable on those presented in Fig. 2.

Isolation of Crystalline DFP-Inhibited Chymotrypsin—A solution of 0.855

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[12] We have recently found that the molecular weight of the chymotrypsin preparation we used is 27,000 ± 1000, rather than 40,000 (13).

gm. of chymotrypsin (= 0.0317 mM of the enzyme) was prepared in 500 ml. of 0.2 M phosphate buffer at pH 7.7. To this, 0.050 mM of DFP was added in two portions with a 30 minute interval. The solution was then stored at 5° for 18 hours, when 98 per cent of the enzyme was found to be inhibited. The protein was precipitated from solution by 0.7 saturation with ammonium sulfate. After filtration the protein was crystallized twice according to the method of Kunitz and Northrop (12) for α-chymotrypsin. Although the resulting crystals were indistinguishable from normal α-chymotrypsin, the crystallization was more complete than with a control sample of α-chymotrypsin. Very little protein remained in solution under the conditions used, and the solubility had been appreciably changed. The inhibited enzyme was then dissolved in approximately 50 ml. of water and dialyzed in cellophane tubing against cold dilute hydrochloric acid at pH 3.5 for several days and then dialyzed exhaustively against water. The aqueous solution was finally lyophilized. Approximately 50 per cent of the protein was recovered. After the dialysis and lyophilization, it was completely inactive, as measured with hemoglobin and with TSAME. The inert (inhibited) protein was also mixed with active α-chymotrypsin in equal amounts and assayed. The activities obtained on both types of substrate were identical to those obtained when no DFP-inhibited chymotrypsin was present. Hence, no excess of DFP was present in the inert protein.

TABLE I

Effect of DFP, TEP, and Parathion on Liver Esterase, Acetylesterase, and Trypsin

Enzyme	pK of inhibition by		
	Diisopropyl fluorophosphate	Tetraethyl pyrophosphate	Parathion
Esterase (liver)*	<6.0	6.0	4.0
Acetylesterase†	4.3	6.0	‡
Trypsin	4.0	‡	‡

*This enzyme preparation was obtained from guinea pig livers. Diacetin was used as a substrate.

†The acetylesterase was a partially purified enzyme preparation obtained from oranges (14). The values are those previously reported (6).

‡The inhibitors in a concentration of 1×10^{-3} M were without effect on these enzymes.

Effect of DFP, TEP, and Parathion on Several Esterases—The effect of these phosphate ester inhibitors was determined on liver esterase, acetylesterase, and trypsin. The results (Table I) show that, at least in the concentrations studied, only DFP inhibited trypsin. Acetylesterase was inhibited only by DFP and TEP, whereas all three inhibitors were effective on liver esterase, but the pK value differed for each inhibitor. The reason for the varied response to the several inhibitors is not apparent.

DISCUSSION

Many differences in the behavior of several esterases to inhibitors of the phosphate-ester class are now apparent. It cannot yet be concluded whether these differences reflect various reactions of the inhibitors, or a different effect of the same reaction on each enzyme. It may be noted, however, that DFP, the most general of these inhibitory phosphates, is not consistent in its effects. With acetylesterase, the inhibition is quite independent of pH; with trypsin or chymotrypsin the inhibition is dependent upon pH.

All of the results reported here are in accord with the hypothesis that the proteolytic and esterolytic activities of trypsin or chymotrypsin reside in the same active centers, except the anomaly of acetyl trypsin, in which the acetylation had reduced the proteolytic but not the ester-splitting activity. We are inclined to believe that some explanation of this behavior in accordance with the hypothesis of identity between ester and protein-splitting centers should be sought. The argument that this acetyl trypsin is not a unit substance does not appear to carry much weight. Whether irregularly acetylated or contaminated by inhibitory impurities, the fact remains that, if only one active group is involved, the involvement should nevertheless apply to both reactions.

It has been shown that the esterase activity of the acetylated protein was about double the proteinase activity, when compared with normal trypsin. The esterase activity of the acetylated protein was also harder to inhibit by DFP than its proteinase activity. In fact the activities could not be *completely* inhibited by DFP in any reasonable concentration.

On the other hand, the evidence (as presented here and also as given by Fraenkel-Conrat *et al.* (7)) indicates rather conclusively that acetyl groups do not combine with the enzyme at the same point as does DFP. The presence of acetyl neither destroys enzymic activity nor prevents subsequent inhibition with DFP. A reasonable explanation of the facts as known is that some of the acetyl groups occupy a strategic position with respect to the proteolytic (and esterolytic) center. By virtue of this position, the approach of ovomucoid to the functional group is prevented and that of hemoglobin considerably restricted, while access of DFP to the functional group is much less impaired and access of ester substrate molecules is not impaired at all. If this represents the true state of affairs, it is obvious that the size of the molecule approaching the functional group is of importance, but not entirely critical; otherwise DFP inhibition should be as easily obtained with acetyl trypsin as with the original enzyme.

It now appears in the case of chymotrypsin and DFP that a definite and probably stoichiometric compound of enzyme and inhibiting ester is formed. It seems to us a good working hypothesis to consider that the enzyme combines with DFP at the same point as it does with a hydrolyzable ester. In one case, however, hydrolysis takes place with subsequent release of the reaction products by the enzyme, while on the other hand the DFP complex could remain unaltered because of the inability of the enzyme to hydrolyze it. Such a combination could be irreversible (as the DFP inhibition is found to be), and the active centers so covered would then be unavailable for esterolysis. Such a hypothesis must include prominently the possibility that DFP combines with the active group in a manner unlike that of a hydrolyzable ester with the esterase (for instance by the elimination of fluorine). This would at least explain the difference in inhibitory effect between DFP and the other phosphates tested. It must be admitted, however, that no positive evidence yet exists that DFP does not react with a neighboring group and thus interfere with the enzyme action by an effect akin to steric hindrance. In any event a complete understanding of the mode of action of DFP on esterases may well lead to an understanding of their mode of action.

SUMMARY

1. DFP was found to be without effect on chymotrypsinogen. After treatment with a relatively large amount of DFP, chymotrypsinogen was again crystallized and then converted to normal chymotrypsin by the action of trypsin.

2. Trypsin was inhibited by minute amounts of DFP. The inhibition was greater at neutrality than at acid pH values. However, because of the instability of trypsin at neutrality, it was necessary to carry out the inhibition studies at pH 5.0. At this pH the inhibition was progressive with time, and was essentially complete in 24 hours at a concentration of DFP of 4×10^{-4} M. The esterase and proteinase activities of trypsin were equally affected, showing that the two activities probably reside in the same active centers of the molecule.

3. Acetyl trypsin, in which most of the amino groups had been acetylated, required higher concentrations of DFP for inhibition, and the inhibition was never observed to be as complete as with trypsin. Acetylation of trypsin caused a decrease in proteinase activity without affecting that of the esterase. In spite of this anomaly, the esterase and proteinase activ-

ities are thought to reside in the same active center of trypsin. The reasons for this hypothesis are discussed.

4. Chymotrypsin is inhibited by DFP. The inhibition is greatest at pH 7.5 to 7.7. The enzyme was 50 per cent inhibited by 8×10^{-6} M DFP. The inhibition of chymotrypsin proceeded much more rapidly than with trypsin. Approximately 1 mole of inhibitor was required for complete inhibition of chymotrypsin. Both α- and β-chymotrypsin were similarly inhibited by DFP. The esterase and proteinase activities were equally inhibited by DFP, showing the probable identity of the two activities.

5. α-Chymotrypsin, completely inhibited by DFP, was crystallized thereafter by the procedure used with the active enzyme. After a recrystallization, dialysis, and lyophilization, the enzyme was still completely inhibited. The crystalline DFP-inhibited chymotrypsin is being used in a study of the mode of action of DFP on enzymes.

6. Of the three inhibitors, DFP, TEP, and parathion, only DFP was inhibitory to trypsin. Both DFP and TEP inhibited acetylesterase, whereas all three were effective on liver esterase.

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MODE OF INHIBITION OF CHYMOTRYPSIN BY DIISOPROPYL FLUOROPHOSPHATE

I. INTRODUCTION OF PHOSPHORUS*

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It has been previously shown that the esterase and proteinase activities of trypsin and chymotrypsin (1, 2) were equally inhibited by diisopropyl fluorophosphate (DFP) (3). With chymotrypsin, 50 per cent inhibition occurred when 0.55 mole of inhibitor per mole of enzyme[1] was used at pH 7.7. On the basis of a linear relationship between the per cent inhibition and the concentration of DFP, 1.1 moles of inhibitor would be needed for complete inhibition. Crystalline DFP-inhibited chymotrypsin was obtained by the reaction of DFP slightly in excess of 1 mole to 1 mole of enzyme. This reaction product remained completely inactive either as an esterase or proteinase after recrystallization, dialysis, and lyophilization. Furthermore, it did not inhibit active chymotrypsin when mixed therewith.

On the other hand, DFP was without effect on crystalline chymotrypsinogen. After treatment with DFP, this zymogen could again be crystallized and converted by the action of trypsin to chymotrypsin. The resulting enzyme was found to be just as active and just as susceptible to inhibition by DFP as that resulting from untreated chymotrypsinogen. From this it was concluded that if there was a reaction between DFP and chymotrypsinogen, it did not affect the conversion to the active chymotrypsin or the inhibition of the resulting enzyme by DFP (3).

By the use of DFP containing radioactive phosphorus (P^{32}) it has now been shown that the phosphorus moiety of DFP is firmly attached to the crystalline DFP-inhibited chymotrypsin. Furthermore, the amount of phosphorus bound (after treatment of chymotrypsin with a small excess of DFP) in the twice crystallized, dialyzed, and completely inhibited enzyme was found to be 1.1 moles per mole of enzyme, a result which is in good agreement with the amount previously calculated on a stoichiometric basis from the 50 per cent inhibition value.

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* Enzyme Research Division Contribution No. 119.

[1] We have recently found that the molecular weight of the chymotrypsin preparation we used is 27,000 ± 1000, rather than 40,000 (4).

Chymotrypsinogen, when treated with radioactive DFP under conditions identical with those used for the chymotrypsin, did not react with the DFP as shown by the failure of the chymotrypsinogen to become radioactive. Therefore, as suggested previously (3), the conversion of the zymogen to the active enzyme liberates not only the groups responsible for activity but also those which react with DFP.

The attachment of the phosphorus moiety of DFP during the inhibition reaction of chymotrypsin is suggestive that the mode of action of DFP is not the same as that of hexaethyl tetraphosphate (HETP). Brauer (5) has found that in the inactivation of human plasma cholinesterase with HETP containing P^{32} no stable combination between the esterase and a phosphorus-containing moiety of HETP took place. He suggested that the mechanism of HETP inhibition involved an exchange reaction between an active hydrogen of the protein and an alkyl group of the inhibitor. Since tetraethyl pyrophosphate (TEP), the active constituent of HETP, was found to be without effect on chymotrypsin (3), it is apparent that the mechanism of DFP and TEP inhibition must differ.

Materials and Methods

The crystalline chymotrypsin[2] (57 per cent enzyme) was obtained as a dry filter cake. The crystalline chymotrypsinogen[3] was recrystallized seven times after receipt, and then exhaustively dialyzed and lyophilized. The proteinase and esterase activities were determined by the methods described previously (3). The radioactive DFP,[4] which contained approximately 1 millicurie per 25 mg., was made up to be 0.0108 M in isopropanol. The radioactivity determinations were made with a Geiger-Müller scaler. All the countings for the chymotrypsin experiment were carried out within a few hours at the end of the experiment. Similarly, the countings of the chymotrypsinogen experiment were completed within a few hours, but at a later date.

EXPERIMENTAL

Inhibition of Chymotrypsin by Radioactive DFP—To a solution of 1 gm. of chymotrypsin preparation (570 mg. or 0.0211 mM of enzyme[5]) in 333 ml. of 0.2 M phosphate buffer at pH 7.7, 3.3 ml. of 0.0108 M radioactive DFP were added. The total count of the reaction mixture was 2.65 × 10^6 per minute. From a separate count of an aliquot of the DFP solution, inactivated with alkali, the count of the amount of DFP added was 2.67 × 10^6 per minute; hence the reaction mixture had the correct count. After incubating overnight at approximately 20°, the esterase activity had been reduced by more than 95 per cent. The reaction mixture was adjusted to pH 4.0 with 5 N H_2SO_4 and the protein precipitated therefrom by 0.8 saturation with ammonium sulfate. The filtrate, which was essentially free of protein, had a total count of 0.74 × 10^6 per minute, or 27.9 per cent of that of the reaction mixture. Hence, 0.025 mM of DFP had reacted with 0.0211 mM of chymotrypsin. Therefore the molar ratio of the phosphate moiety of the DFP combined with the chymotrypsin was 1.2. The protein filter cake was then crystallized by the method used for the active enzyme (6). After one recrystallization the inert protein crystals were indistinguishable in form from those of active α-chymotrypsin. The crystalline product was dissolved in 30 ml. of water and dialyzed against dilute HCl at pH 3.5 for 4 days in the cold, the dilute HCl solution being changed daily. After the 1st day the HCl solution had a negligible radioactivity. The inert protein solution was then dialyzed against cold 0.01 M phosphate buffer at pH 3.5 for 1 day longer. The resulting protein solution, which was still completely inactive, had a count of 2.48 × 10^4 counts per minute per ml. and a nitrogen content of 1.32 mg. per ml. The count corresponded to 3.31 × 10^{-4} mM of DFP and the nitrogen to 3.08 × 10^{-4} mM of protein. Hence the molar ratio of DFP to chymotrypsin in this final product was 1.08:1. Therefore, it is clear that the phosphorus moiety of the DFP was introduced into the chymotrypsin and that the amount of phosphorus so introduced corresponded to the amount of DFP necessary to cause complete inhibition of the chymotrypsin (3).

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[2] Purchased from the Armour Laboratories, Armour and Company, Chicago, Illinois.

[3] Purchased from the Plaut Research Laboratory, Lehn and Fink Products Corporation, Bloomfield, New Jersey.

[4] Obtained through the courtesy of Dr. Bernard J. Jandorf, Biochemistry Section, Medical Division, Army Chemical Center, Edgewood, Maryland.

[5] The nitrogen content of chymotrypsin is 15.83 per cent (4).

Failure of Radioactive DFP to React with Chymotrypsinogen—A solution of crystalline chymotrypsinogen (500 mg. in 333 ml. of 0.2 M phosphate buffer at pH 7.7) was treated with 3.3. ml. of 0.0108 M radioactive DFP. After standing for 24 hours at approximately 20° the pH of the solution was brought to 4.8 by the addition of 2 M H_2SO_4. The protein was precipitated by 0.8 saturation with ammonium sulfate. After filtration an aliquot of the protein-free filtrate and an aliquot of the DFP solution, inactivated by alkali, were examined for radioactivity. The total count of the filtrate was found to be 1.88 × 10^6 per minute and that of the DFP added was 1.97 × 10^6 per minute. Hence, less than 5 per cent of the DFP was taken up by the chymotrypsinogen, whereas chymotrypsin under the same conditions would have reacted with approximately 70 per cent of the DFP. Therefore there is very little, if any, reaction between chymotrypsinogen and DFP.

SUMMARY

By the use of radioactive DFP it was found that the phosphorus of DFP was introduced into crystalline chymotrypsin by the inhibition reaction of DFP on chymotrypsin. The amount of phosphorus introduced was 1.1 moles per mole of enzyme. This amount of phosphorus corresponds to the amount of DFP previously found necessary to inhibit chymotrypsin completely. Chymotrypsinogen under the same conditions did not react with DFP. Hence the conversion of the zymogen to the active enzyme liberates not only the groups responsible for activity but also those with which DFP reacts, the two possibly being the same.

It is suggested that the mode of action of DFP may well differ from other phosphate ester inhibitors.

We are indebted to Dr. Bernard J. Jandorf of the Medical Division, Army Chemical Center, Edgewood, Maryland, for supplying us with the radioactive DFP used in this work and to Dr. Bernard Axelrod of the Enzyme Research Division, for making the counts of radioactivity.

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CALCIFICATION OF TEETH

III. X-RAY DIFFRACTION PATTERNS IN RELATION TO CHANGES IN COMPOSITION*

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PLATES 1 AND 2

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The purpose of the present investigation was to study the x-ray diffraction patterns of teeth in relation to changes in composition induced by diet.

Previous studies showed that the composition of the upper incisors of the albino rat can be influenced by the Ca:P ratio of the diet (1). In a typical experiment the mean values of the $PO_4:2CO_3$ ratios for enamel (used as an index of *n* in the apatite formula $[Ca_3(PO_4)_2]_n[CaCO_3]$) varied from 3.71 on the high Ca:P diet to 7.72 for the low Ca:P diet, and for the corresponding dentins the mean values of these ratios varied from 5.47 to 9.31. There was no significant influence of the diet on the $Ca:PO_4$ ratios of the teeth. The residual $Ca:PO_4$ ratios of the enamel were higher and those of the dentin lower than the theoretical 1.50 in the apatite formula $[Ca_3(PO_4)_2]_n[CaCO_3]$.

In the present study, the compositions of the enamels and dentins obtained (Table I) are in essential agreement with results obtained previously; namely, for rats fed the high phosphorus-low calcium diet (Diet C), the $PO_4:2CO_3$ ratios of both enamel and dentin are higher than the corresponding ratios for rats fed the low phosphate-high calcium diet (Diet B) (1). The $Ca:PO_4$ ratios of all enamels are higher than for the corresponding dentins.

For purposes of comparison, enamel and dentin of young rats (of the same age as the experimental animals), raised on our stock diet (2), were analyzed (Table I). The mean value of the $Ca:PO_4$ ratio of the enamel in Experiment 1 is 1.47 and in Experiment 2, 1.53. This is distinctly lower than any ratio we observed for the enamel of rats on the experimental diets, both in the present study and in the previous investigation in which the relation of the composition of blood, teeth, and diet was established

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(1). The $Ca:PO_4$ ratio for dentin is 1.20 for Experiment 1 and 1.21 for Experiment 2. These are also lower than the values obtained for the dentin of animals on the experimental diets (1).

Results show that prior to ignition *the predominant x-ray diffraction pattern is that of apatite in all cases,* the lines being more distinct in the enamel than in the dentin (Table I; Figs. 1 and 2). This distinctness of lines has been observed previously and is due to the lower percentage of organic matter in the enamel (1, 3–8). The fact that *the predominant pattern is that of apatite, in spite of wide variations of composition,* can be interpreted to mean either that the crystalline form is the same regardless of composition or that x-ray powder diagrams cannot reveal fine differences in crystalline structure. The persistence of apatite as the main solid structure of teeth has been shown before (9, 10). This was true even when considerable changes in composition took place under the influence of sodium fluoride (11). The situation seems to be analogous to that of bone (12) in which, in spite of wide variations in composition, all x-ray evidence to date (9, 10) indicates that bone salts are present in an apatite structure.

TABLE I

X-Ray Diffraction Patterns of Teeth of Various Compositions

Diet	Serum		Specimen	$PO_4:2CO_3$	$Ca:PO_4$	X-ray patterns	
	$PO_4:CO_3$	$Ca:PO_4$				Before ignition	After ignition
	molar ratio	*molar ratio*		*molar ratio*	*molar ratio*		
B	0.019	6.91	Enamel	4.01	1.70	Apatite	Apatite
			Dentin	4.55	1.35	"	β-$Ca_3(PO_4)_2$
C	0.093	0.615	Enamel	5.40	1.67	"	Apatite
			Dentin	7.50	1.39	"	β-$Ca_3(PO_4)_2$
Stock. Experiment 1	0.112	1.10	Enamel	5.21	1.47	"	"
			Dentin	6.15	1.20	"	"
Experiment 2	0.121	1.09	Enamel	8.34	1.53	"	"
			Dentin	12.01	1.21	"	"

Composition of Diets

Diet	Calcium	Phosphorus	Ca:P
	per cent	*per cent*	*molar ratio*
B	1.20	0.203	4.60
C	0.028	0.841	0.026
Stock. Experiment 1	0.369	0.398	0.72
" " 2	0.282	0.380	0.58

There are a number of factors that may account for the predominance of the apatite structure in spite of wide variations in composition. Apatite may be considered a continuous series of solid solutions in which the composition of the solid reflects the composition of the liquid with which it is in equilibrium (9). Ionic exchange between the liquid and solid phase has been indicated (13) and may be a cause of further changes in composition. Adsorption is an added factor that must be considered in accounting for the variation in the composition of tooth salts (3, 14, 15). The experiments of Logan and Taylor (16) with inorganic models indicate that the calcium carbonate portion of the tooth may be adsorbed or at least be present in higher concentrations on the surface than in the interior of the tooth. Walden and Cohen (17) have shown that the constituents adsorbed on the surfaces of crystals cannot be detected by means of x-ray powder diffraction.

After ignition, the dentin of all groups and the enamel of the group on the stock diet showed β-$Ca_3(PO_4)_2$, while the enamel of animals on both the low calcium-high phosphorus diet and the high calcium-low phosphorus diet continued to give apatite patterns (Table I; Figs. 1 and 2). *The occurrence of β-$Ca_3(PO_4)_2$ on ignition appears to be a function of the Ca:PO_4 ratios.* Hodge and coworkers (18), working with calcium phosphate precipitates of varying composition, showed that when the Ca:PO_4 ratio[1] was less than 1.50 the pattern on ignition was always, that of β-$Ca_3(PO_4)_2$. When the ratio was between 1.50 and 1.59, the pattern was predominantly that of β-$Ca_3(PO_4)_2$, and when above 1.59, the ignited specimen continued to give apatite patterns. If one reexamines the data of Dallemagne and Brasseur (19), it is seen that bone, which after treatment with KOH in glycerol had a Ca:PO_4 ratio of 1.73,[1] on ignition gave an apatite pattern. After treatment with hydrochloric acid (which removed the carbonate) and washing with water, the residue of the original bone had a Ca:PO_4 ratio of 1.50 and on ignition gave a β-$Ca_3(PO_4)_2$ pattern. Similarly pure $Ca_3(PO_4)_2$, which they prepared with an actual ratio equal to the theoretical of 1.50, gave β-$Ca_3(PO_4)_2$ on ignition. When this substance was hydrolyzed with alkali, the resulting compound had a ratio of 1.67 and gave the apatite pattern on ignition. When this precipitate was treated with hydrochloric acid so that the residue once more had a ratio of 1.50, and then ignited, the β-$Ca_3(PO_4)_2$ pattern was again obtained. Hirschman and his coworkers (20) showed that when commercial apatite with a Ca:PO_4 ratio of 1.67 was ignited the apatite pattern was obtained. When this was mixed with $CaHPO_4$ so that the resulting mixture had a Ca:PO_4 ratio of 1.51 and then ignited, the β-$Ca_3(PO_4)_2$ pattern predominated. Thus it appears that the Ca:PO_4 ratio of such compounds is the predominant factor that decides whether on ignition one obtains a β-$Ca_3(PO_4)_2$ or an apatite pattern. The pattern of the ignited product may therefore be used as an indirect index of the $Ca:PO_4$ ratio.

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[1] Weight ratios given by the authors are converted to molar ratios.

In connection with the analyses obtained for the rats on the stock diet of Bills *et al.* (2), it may be worth referring to the discussion in our earlier paper (1), where it was postulated that, while the $PO_4:CO_3$ ratio of the blood serum will have an influence on the composition of the tooth on a given diet, the components of the diet, other than calcium and phosphorus, are likely to influence the type of relationship that will be found between blood and teeth. On comparing all results in Table I it is seen that although in Experiment 1, on the stock diet, there is a slight deviation, the $PO_4:2CO_3$ ratios of enamel and dentin change in the same direction as do serum $PO_4:CO_3$ ratios.

Both enamel and dentin of the group on the stock diet have distinctly lower $Ca:PO_4$ ratios than those obtained for the corresponding enamel and dentin in any of the experimental groups (Table I) (1). These differences cannot be due to the Ca:P ratio of the stock diet, since this ratio was covered by the range of ratios in the experimental Diets B, C, and D (1). They might be due to other differences between the experimental and the stock diets. The main ingredients of the Bills stock diet (dried milk, crude casein, whole yellow corn, alfalfa, and cottonseed meal) are different from those of the experimental diets (degerminated yellow corn-meal, wheat gluten, and brewers' yeast) (1, 2). Thus differences in $Ca:PO_4$ ratios for the enamel and dentin of the rats on the stock diet and those on the experimental diets (Table I) may be accounted for by differences between the composition of the stock diet and experimental diets other than calcium and phosphorus.

The difference in the $PO_4:2CO_3$ ratios of the enamel and dentin of the two groups on the stock diet raises a similar question. In the experimental diets the same batch of ingredients was used throughout a given experiment. Only the calcium and phosphate content was changed by the addition of salts. For the stock diet, however, different batches of the ingredients were used in the two experiments carried out 6 months apart. Each one of the main ingredients can undergo variation from batch to batch and the composition of two stock diets may therefore be different.

It must be added that the possibility that seasonal variations cause changes in the composition of teeth cannot be excluded. Blincoe *et al.* (21) have shown that the calcium and inorganic phosphorus of serum are influenced by environmental temperature.

A systematic study of the influence of not only the Ca:P ratios of the diet but also that of other components (vitamins, proteins, fats, carbohydrates, and trace minerals) may throw further light on the subject.

EXPERIMENTAL

Young rats, of an original Wistar strain, 21 to 23 days of age, kept on the Bills stock diet (2), were weaned and placed on one of the experimental diets. Diets B and C were essentially the same as that described before (1) except that the basal diet (used in making up the experimental diets) had 0.20 per cent phosphorus compared to 0.118 per cent in the previous basal diet (1). The stock group was fed the Bills diet (2). The calcium and phosphorus content of the diets is given in Table I.

Forty-eight animals were placed on Diet B, forty-nine on Diet C, and a total of 58 on the stock diet (twenty-two in Experiment 1, and, 6 months later, thirty-six in Experiment 2). The animals were sacrificed at the end of 45 days and the blood and teeth from the rats in each group were pooled, sampled, and analyzed as previously described (1). Mean values for duplicate analyses of replicate samples are reported in Table I.

Powder diffraction patterns were made on both ignited and unignited specimens, ground to pass a 250 mesh sieve, by means of a 57.3 mm. Debye-Scherrer camera (22). The samples were mounted in thin walled capillaries and nickel-filtered copper radiation was used. Exposures were of 3½ hours duration. Ignition was conducted in platinum crucibles for 2½ hours at 900°.

SUMMARY

1. X-ray powder diffraction patterns of enamel and dentin of widely varying composition gave apatite as the dominant pattern. The teeth used were the upper incisors of albino rats of the Wistar strain.

2. After ignition at 900°, the predominant pattern was apatite when the Ca:PO$_4$ ratio was more than 1.60 and was β-$Ca_3(PO_4)_2$ when the ratio was 1.53 or less. These findings are in harmony with those obtained by other investigators using inorganic calcium phosphate.

3. The pattern obtained after ignition at 900° appears to be an index of the Ca:PO$_4$ ratio.

4. It was again possible to show a relationship between PO$_4$:2CO$_3$ ratios of enamel and dentin and PO$_4$:CO$_3$ ratios of serum.

5. The differences in Ca:PO$_4$ ratios of the enamel and dentin obtained from animals on experimental diets and the animals on the stock diet suggest the importance of examining the influence of not only the Ca:P ratios of the diets but also that of the other components (vitamins, proteins, fats, carbohydrates, and trace minerals).

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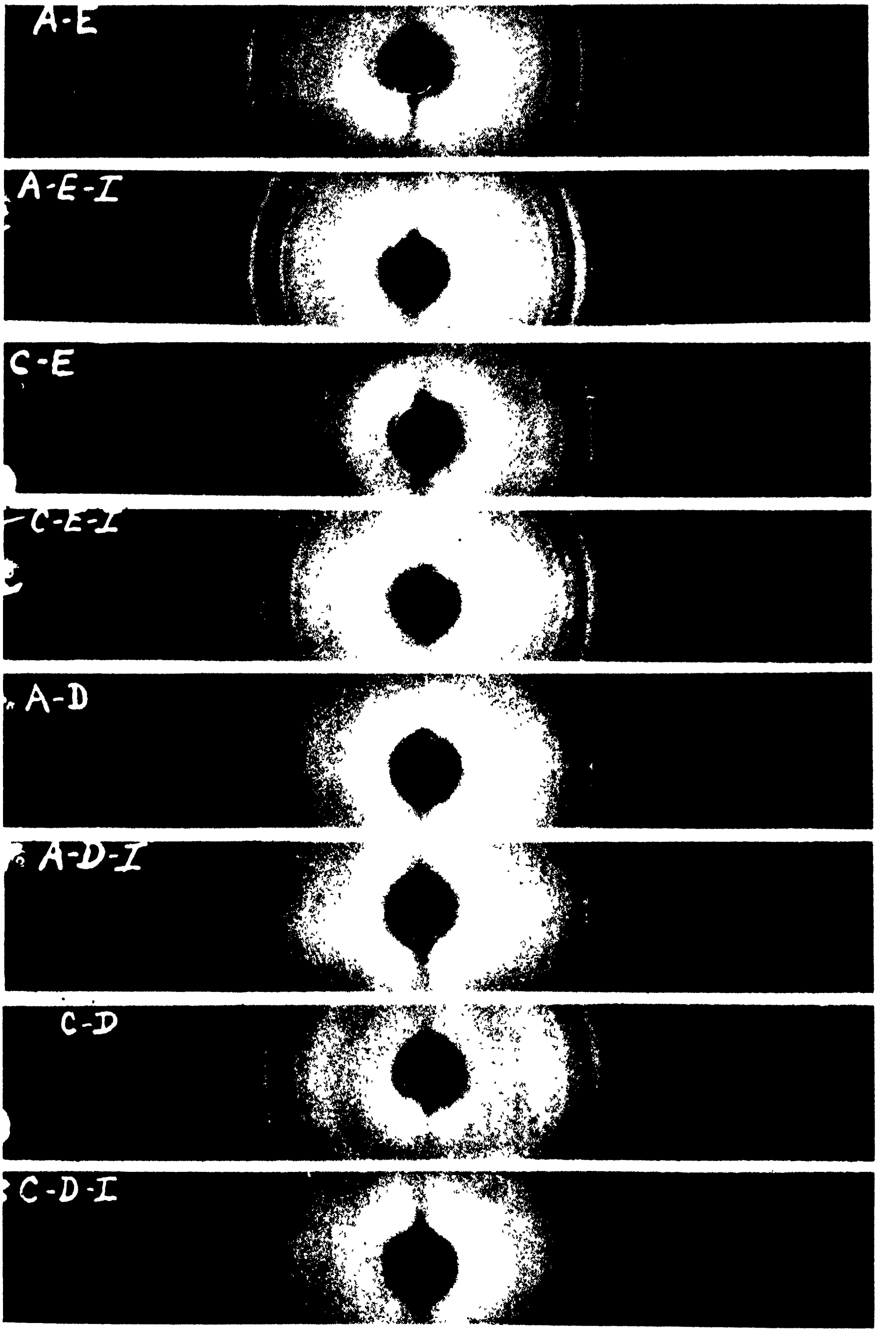
EXPLANATION OF PLATES

PLATE 1

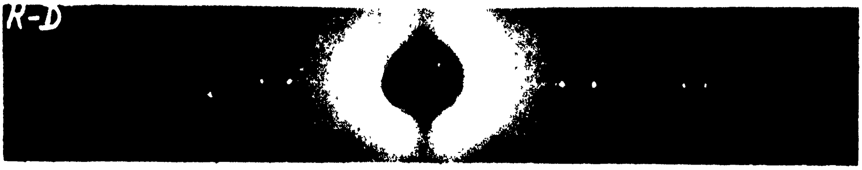
FIG. 1. X-ray diffraction patterns of teeth from rats on Diets B and C. *A*, Diet B; *C*, Diet C; *D*, dentin; *E*, enamel; *I*, ignited at 900°.

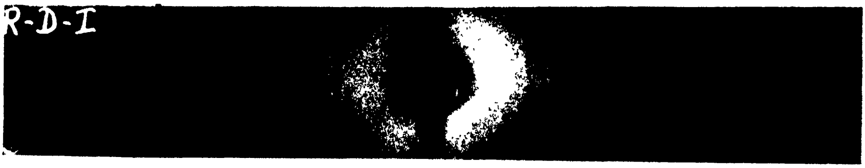
PLATE 2

FIG. 2. X-ray diffraction patterns of teeth from rats on stock diet. *R*, rat; *E*, enamel; *D*, dentin; *I*, ignited at 900°.



(Sobel, Hanok, Kirshner, and Fankuchen, Calcification of teeth)





(Sobel, Hanok, Kirshner, and Fankuchen, Calcification of teeth)

URINARY PHENOLS

IV. THE SIMULTANEOUS DETERMINATION OF PHENOL AND *p*-CRESOL IN URINE*

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Phenol and *p*-cresol constitute the volatile urinary phenols. The relative amount of each compound, however, is not definitely known, since the reported values are based on unreliable or non-specific methods (1–3). The inadequacy of these methods has been discussed recently by Warshowsky and Schantz (4). These workers determine phenol and *m*-cresol in bacteriological materials by a counter-current distribution technique. The present paper describes a simple, differential, colorimetric method for the simultaneous determination of phenol and *p*-cresol in urine. It consists in the determination of the total phenolic value both as phenol and as *p*-cresol by two different colorimetric procedures. Simultaneous equations arising from these determinations are readily solved because the ratios of the values for phenol to *p*-cresol given by each procedure differ considerably. These ratios were obtained by the analysis of aqueous solutions of phenol and *p*-cresol of known concentrations. A study of absorption spectra of chromogens produced by phenol and *p*-cresol with various reagents, including studies reported in the literature (4–5), indicated that the method of determining components of a binary mixture by measurements at two appropriately chosen wave-lengths was not feasible.

EXPERIMENTAL

Analysis of Phenol-p-Cresol Mixtures Prepared from Known Solutions—After considerable preliminary experimentation, procedures utilizing the Folin-Ciocalteu reagent (6) and diazotized sulfanilic acid (7) were adopted. Aqueous solutions, and also urines, containing varying amounts of purified phenol and *p*-cresol were analyzed with these reagents according to the distillation procedure of Volterra (8) and the ether extraction method of Schmidt (9). In the distillation method measured amounts of the phenol and *p*-cresol solutions were mixed and diluted with water to 200 ml. The pH was adjusted to 1 with sulfuric acid. The solution was refluxed for 30 minutes in an all-glass apparatus and then distilled. Six 100 ml. fractions of distillate were collected. Water was added during the distillation.

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About 80 per cent of the phenols came over in the first two fractions. 1 ml. of the Folin-Ciocalteu reagent (diluted 1:1 with water) and 2 ml. of 20 per cent sodium carbonate were added to 10 ml. of pooled distillate. A blank was also prepared. The tubes were placed in boiling water for exactly 1 minute and then immediately cooled (8). The blue solutions were read in a Klett-Summerson photoelectric colorimeter which contained the red light filter No. 66 (640 to 700 mμ). The instrument was first adjusted to zero with the blank.

In the second colorimetric procedure 5 ml. of alcohol and 3 ml. of freshly distilled ether were added to 2 ml. of pooled distillate, and also to 2 ml. of water, followed by 1 ml. of a cold solution of diazotized sulfanilic acid and 1 ml. of 1 per cent sodium carbonate.[1] The yellow-orange solutions were read in the photoelectric colorimeter which now contained a blue light filter No. 42 (460 to 465 mμ) against the blank set at zero.

TABLE I

Recovery of Phenol and p-Cresol Added to Water and to Urine

The values are measured in mg. per 1000 ml.

Medium	Compound added		Compound recovered			
			Distillation method		Ether extraction method	
	Phenol	*p*-Cresol	Phenol	*p*-Cresol	Phenol	*p*-Cresol
Water	5	100	5.9	102	5.1	104
" . . .	3	60	3.5	58	3.2	57
"	10	200	10.8	214	11.8	207
"	25	25	22.0	28	20.0	27
" . . .	25	25	28.3	26	27.5	23
Urine . . .	10	125	11.6	112	10.7	129
" .	10	50	12.5	55		
" . . .	10	50	8.4	56		

In the ether extraction method, a measured volume of the phenol-*p*-cresol hydrolysate was adjusted to pH 10 with sodium hydroxide and extracted with ether for 4 hours, as previously described (9). 3 ml. of the ether extract were shaken in a test-tube with 1 ml. of 0.1 N sodium hydroxide and a trace of antibumping powder (prepared by pulverizing a porcelain evaporating dish). The ether was evaporated by immersion of the tube in hot water. Then 1.3 ml. of 0.1 N sulfuric acid and 7.7 ml. of water were added and the phenolic values determined with the Folin-Ciocalteu reagent as usual. A 3 ml. aliquot of the ether extract was also analyzed for phenol and for *p*-cresol with the sulfanilic acid reagent, as outlined above. The values given by each method were obtained from standard curves developed from appropriate solutions of phenol and *p*-cresol.

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[1] This reagent was prepared by adding 3 ml. of cold 5 per cent sodium nitrite to 4 ml. of a cold solution of sulfanilic acid (4.5 gm. of sulfanilic acid and 45 ml. of hydrochloric acid per 500 ml. of solution). 5 minutes later an additional 3 ml. of the sodium nitrite solution were added. After 15 minutes the solution was diluted to 50 ml. with water and kept cold. The reagent was prepared fresh daily. The sodium carbonate solutions were prepared from the recrystallized salt.

The colors given by each reagent are stable and obey Beer's law. It was found that 1 part of phenol has the chromogenic power of 1.66 parts of *p*-cresol with the Folin-Ciocalteu reagent and 12 parts of *p*-cresol with the sulfanilic acid reagent under the experimental conditions described above. These ratios remain constant regardless of the concentration of the phenolic bodies. The intensities of the colors given by phenol and *p*-cresol in both colorimetric procedures were found to be additive for all binary mixtures.

TABLE II

Analysis of Eleven 24 Hour Specimens of Normal Human Urine

The values are measured in mg. per 24 hour specimen.

Urine No.	Sex	Distillation method		Ether extraction method	
		Phenol	*p*-Cresol	Phenol	*p*-Cresol
1	Male	9.1	90	10.8	87
2	"	10.0	81	12.7	78
3	"	13.0	100	12.5	98
4	"	11.6	82	11.8	80
5	"	9.6	80	12.5	71
6	"	11.0	100	10.4	95
7	"	8.0	64	8.3	65
8	"	9.7	108	9.1	117
9	Female	8.9	71	9.2	75
10	"	7.8	83	8.3	81
11	"	10.0	100	9.7	103
Maximum		13.0	108	12.7	117
Minimum		7.8	64	8.3	65
Average		9.88	87.2	10.48	86.4

Sample Calculation—A prepared mixture containing 10 mg. of phenol and 200 mg. of *p*-cresol per 1000 ml. was subjected to analysis as described above. The values obtained were 325 mg. as *p*-cresol or 27 mg. as phenol with the sulfanilic acid reagent and 213 mg. as *p*-cresol or 138 mg. as phenol with the Folin-Ciocalteu reagent per 1000 ml. Since the chromogenic ratios of phenol to *p*-cresol are 1:12 and 1:1.66, respectively, the following equations arise:

$$\begin{array}{rll} 12 \text{ phenol} + p\text{-cresol} & = 325 & \text{(sulfanilic acid reagent)} \\ 1.66 \quad \text{``} \quad + \quad \text{``} & = 213 & \text{(Folin-Ciocalteu reagent)} \\ \text{Phenol} & = 10.8 & \text{(phenol added, 10.0)} \\ \text{Phenol} + \dfrac{p\text{-cresol}}{12} & = 27 & \text{(sulfanilic acid reagent)} \\ \text{``} \quad + \dfrac{p\text{-cresol}}{1.66} & = 138 & \text{(Folin-Ciocalteu reagent)} \\ p\text{-Cresol} & = 214 & (p\text{-cresol added, 200}) \end{array}$$

The data are given in Table I and indicate that satisfactory recovery of phenol and *p*-cresol added either to water or to urine can be accomplished by the procedures described.

Analysis of Urines for Phenol and p-Cresol—24 hour urine specimens were collected from healthy individuals who were on an average mixed diet. 50 ml. of the urine were diluted to 200 ml., adjusted to pH 1 with sulfuric acid, and refluxed for 30 minutes. Distillation and analysis for phenols were performed as outlined above. In the ether extraction method, urine hydrolysis and extraction were conducted as previously described (9). Analyses of the ether extracts were effected as outlined above. The data are given in Table II. The average value found for phenol was 9.88 mg. and for *p*-cresol 87.2 mg. by the distillation method, and 10.48 mg. and 86.4 mg., respectively, by the ether extraction method for the eleven specimens. Hence values given by the two methods are in satisfactory agreement. The data indicate that *p*-cresol constitutes approximately 90 per cent of the volatile urinary phenols. Difference in sex did not influence the values in the specimens analyzed. There is some evidence that a portion of the urinary *p*-cresol may be a product of sex hormone metabolism (3). The proportion of phenol to *p*-cresol in the various specimens remained relatively constant.

Obviously the method is limited to those binary mixtures in which *p*-cresol predominates. This fact, however, makes it especially well adapted to urine analysis. The method can undoubtedly be extended to other complex media in which an analogous situation exists. The writer is indebted to Dr. G. S. Weiland and Dr. Marie Andersch for helpful suggestions.

SUMMARY

A simple, differential colorimetric method is described for the simultaneous determination of phenol and *p*-cresol in urine. It was found that *p*-cresol constitutes about 90 per cent of the volatile urinary phenols.

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STUDIES OF ARTERIOVENOUS DIFFERENCES IN BLOOD SUGAR*

III. EFFECT OF INSULIN ADMINISTERED INTRAVENOUSLY IN THE POSTABSORPTIVE STATE

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In experiments previously described (1) we have shown that in healthy persons hypoglycemia elicits an abrupt shrinkage in the difference between arterial and venous blood sugar levels (A-V difference), a change which indicates a sudden decrease in the rate of peripheral (extrahepatic) glucose assimilation. This observation was made when the subjects were in a state of "spontaneous" hypoglycemia, which appears with regularity as a sequel to the hyperglycemia following glucose feeding. It is generally understood that in this process alimentary hyperglycemia stimulates the secretory activity of the islands of Langerhans, and the increased insulin supply not only checks hyperglycemia, but shoots beyond the goal and produces hypoglycemia.[1] Since this hypoglycemia represents increased insulin action, and insulin action is known to increase A-V differences, it was rather unexpected when we found a contrary effect, *i.e.* a sharp decrease in A-V differences, as soon as a hypoglycemic state[2] set in.

We interpreted this phenomenon as the function of insulin-antagonistic endocrine organs which are excited by hypoglycemia to increased activity. The insulin-antagonistic (diabetogenic) effect of endocrine factors, like anterior pituitary extracts (APE), adrenalin, and cortical hormones,

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[1] Soskin and Allweiss rejected this concept on the basis of their observation that the hypoglycemic sequel to alimentary hyperglycemia can occur under conditions which preclude any increase in the insulin supply (2). They apparently failed to take into consideration the lack of any stoichiometric relationship between insulin supply and insulin action; in particular, they disregarded the fact that the action of one and the same amount of insulin increases with increasing glucose concentrations. (This disparity between insulin supply and insulin action is the subject of the last section of the present paper.)

[2] Let us reiterate here that we use the term hypoglycemia in its physiological meaning, in contradistinction to the conventional (especially clinical) usage in which hypoglycemia means a condition associated with certain alarm reactions, conditions that are precipitated only by intensive hypoglycemic states. In our terminology hypoglycemia denotes any, even the slightest, decline of the arterial blood sugar below its normal postabsorptive level, since the physiological reactions with which we are concerned are precipitated by changes as small as 1 to 3 mg. per cent (1).

is customarily attributed to their action on the liver, and on the liver alone, the consensus being that they raise the glycemic level by increasing the rate of hepatic glycogenolysis. The question may be raised, therefore, whether the insulin-antagonistic effects which we measured in the peripheral tissues can be reasonably attributed to the same known diabetogenic factors. A number of older studies furnish an affirmative answer. Marks (3), for instance, has shown, 16 years ago, that, while glycogen formation in muscle tissues is augmented by insulin injection, this response is prevented by the simultaneous injection of APE. Bennett and Roberts (4) showed, in experiments on eviscerated animals, that the hypoglycemic effect of injected insulin is considerably inhibited by simultaneously administered APE. Both of these experiments demonstrated, at different phases of the process, the same fact as our determination of A-V differences, namely an inhibition of peripheral insulin action by APE factors. This was the basis of our assumption that inhibition of peripheral glucose assimilation under the impact of hypoglycemia (as reflected in our studies in decreased A-V differences) is engineered by insulin-antagonistic factors (*par excellence* by the pituitary-adrenal mechanism) which are stimulated by hypoglycemia. Bouckaert and de Duve (5) also made the assumption that hypoglycemia antagonizes insulin action by stimulating the "orthosympathic system, adrenalin, cortin, anterior pituitary hormones, etc." This concept gained direct support by a recent observation of Gershberg and Long (6), who found that insulin hypoglycemia entails a sharp decrease in the ascorbic acid content of the adrenal glands, a characteristic result of an increased supply of ACTH (adrenocorticotropic hormone of the pituitary). The ACTH, however, is by no means the sole factor in the defense mechanism against hypoglycemia. That the hypophysis supplies potent insulin-antagonistic factors besides the ACTH is sufficiently proved by the fact that the glycogen content of the liver during insulin hypoglycemia is rapidly depleted even in adrenalectomized animals (7). Insulin-antagonistic action under these conditions is undoubtedly tied up with pituitary activity.

Identification of the insulin-antagonistic factors is of great interest, but is outside the scope of the present report; our emphasis is on the fact which we have demonstrated, namely that hypoglycemia activates a defense mechanism which inhibits insulin action in the peripheral tissues. This self-limiting (one is inclined to call it suicidal) sequel to insulin action is not only of theoretical interest, but has an important bearing on clinical insulin therapy. In an earlier communication (8) we attempted to call attention to this practical aspect of the problem.[3] In the present paper

[3] On the basis of extensive observations, we pointed out in that paper the "diabetogenic effect" of hypoglycemia as follows: "Hypoglycemias, caused by over-

we report the results of further experiments which are to answer the question as to whether or not hypoglycemia, produced by the intravenous injection of insulin, mobilizes insulin-antagonistic factors, as does spontaneous hypoglycemia.

EXPERIMENTAL

The subjects in these experiments were, unless otherwise stated, young persons, hospital interns and laboratory workers between 20 and 40 years of age, without any known impairment of their health. With few exceptions they were subjected to glucose tolerance tests in order to ascertain that their carbohydrate metabolism was normal.

The determination of arteriovenous (A-V) differences, as a means of measuring changes in the rate of peripheral glucose assimilation, was carried out with the precautions and accuracy previously described (9). Numerous publications dealing with the subject amply justify the emphasis we place on the accuracy of analytical procedures. A few workers, as for instance Cori and Cori (10) and Himsworth (11), fully aware of this requirement, obtained results which furnished clear cut, valuable information. These investigators used the original Hagedorn-Jensen method with adequate craftsmanship and both found that insulin action increases A-V differences. They found, furthermore, always higher sugar concentrations in arterial than in venous blood.

The majority of subsequent workers seems to have been unaware of the importance of analytical craftsmanship and, undoubtedly on this account, reported findings in which the glycemic levels were often higher in the venous than in the arterial blood (12, 13). Completely oblivious of the conflict between their results and those of the authors just mentioned, these workers, as for example Griffiths (13), attempted to explain their negative (inverted) A-V differences with highly speculative, mostly arbitrary theories.

Insulin was administered intravenously, in the postabsorptive state. We used rather small doses, from 3 to 6 units, in order to avoid, as far as possible, the stress of protracted hypoglycemic reactions. Such stress must be held to a minimum, because its effects overshadow finer details and blot out individual differences in the response of the subjects. In this respect we were greatly influenced by Himsworth's procedure, who, by virtue of using as small doses of insulin as 2.5 to 5 units, produced much valuable information concerning the relationship between dietary factors and

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doses of insulin, entail in the diabetic patient excessive degrees of hyperglycemia and glycosuria. Recurrence of this sequel over considerable periods of time progressively increases the instability of the patient and aggravates the disease."

insulin action, information that would have been completely obscured by larger insulin doses.

Like in Himsworth's studies, the amount of insulin injected was independent of the body weight, although our several subjects varied between 60 and 75 kilos, and one of them weighed 91 kilos. Close adjustment of the insulin dose to body weight, a widely followed precept, implies acceptance of the still surviving misconception that some sort of stoichiometric relationship exists between insulin supply and the amount of glucose "utilized" or "burned." Much factual evidence, inclusive of our own observations to be described later on, proves this "unitarian" concept as completely untenable. There are factors other than the insulin supply itself which greatly affect insulin action. These factors vary from one person to another, and thus it is understandable that healthy individuals of the same body weight show great differences in their response to identical doses of insulin. These differences show up, however, only when the insulin dose is small, for large doses, steam roller-like, simply override existing individual differences.

Extrahepatic Responses to Hypoglycemia

Six subjects were injected intravenously with 5 units of insulin in the morning, 10 to 14 hours after their last meal, and changes in their arterial (capillary) and venous blood sugar levels were determined at 30 minute intervals. The results are recorded in Table I. The response to insulin action, as may be seen, varied considerably from individual to individual, in respect to both the degree of hypoglycemia and the changes in A-V difference. The same individual, however, as demonstrated in Subject 4, showed the same individual characteristics in his response to repeated tests with the same dose. The lowest hypoglycemic level, 27 mg. per cent, was produced in Subject 3, while in Subject 6 (who weighed 1 kilo less than Subject 3) the fall of the blood sugar stopped at 56 mg. per cent. Notable variations were found in the effect of insulin on the A-V difference. In this respect Subjects 1 and 4, on the one extreme, and Subjects 3 and 5, on the other, represent two different types. The first type showed no significant changes, no increase in the A-V difference, whereas in the second type the A-V difference increased appreciably 30 minutes after the injection of insulin. Subject 6 took an intermediate place. Finally, Subject 2 who showed a measurable contraction of the A-V difference after 30 minutes, but then a delayed but significant expansion 90 minutes after injection, fell in still another category.

Such diversity in individual responses to insulin is entirely incomprehensible on the basis of the oversimplified "unitarian" concept, but can be readily explained if insulin action is evaluated as the resultant of a continuous dynamic interplay between two opposing forces, namely insulin on the one side and its antagonists on the other. With this approach we interpret our findings as follows: The onset of insulin-hypoglycemia, which follows directly after intravenous injection, immediately induces the mobilization of the antagonistic factors, the forces of defense against hypoglycemia. This response is inevitably subject to individual variations. In the first place, one must think of quantitative differences, taking it for granted that one individual can produce greater supplies of insulin-antagonistic hormones than another. Secondly, there are differences in the chronology of the response to excitation in the endocrine organs: in one case the reaction will be prompt and resilient; in another instance it will be more or less sluggish and, in consequence, some lag will appear between stimulus and response.

TABLE I

Changes in Total and Extrahepatic Glucose Assimilation after Intravenous Injection of 5 Units of Insulin

The blood sugar (B. S.) and arteriovenous differences (A-V) are expressed as mg. per 100 cc. of blood. The arterial blood sugar was determined in capillary (finger) blood.

Subject No.	Origin of blood	Time after injection of insulin									
		Fasting		0.5 hr.		1 hr.		1.5 hrs.		2 hrs.	
		B. S.	A-V	B. S.	A-V	B. S.	A-V	B. S.	A-V	B. S.	A-V
1	Arterial	96.7		44.8		80.5		84.5		90.5	
	Venous	93.2	3.5	41.3	3.5	75.6	4.9	80.8	3.7	85.1	5.4
2	Arterial	81.5		49.7		73.7		87.5		83.7	
	Venous	74.5	7.0	46.2	3.5	69.7	4.0	75.1	12.4	82.4	1.3
3	Arterial	91.8		50.4		80.5		81.0		81.3	
	Venous	90.5	1.3	37.3	13.1	72.6	7.9	77.0	4.0	79.9	1.4
4	Arterial	89.6		55.4		89.6		91.2		90.7	
	Venous	87.8	1.8	50.8	4.6	88.2	1.4	89.4	1.8	88.6	2.1
4a	Arterial	88.3		62.4		84.0		86.4		86.4	
	Venous	84.3	4.0	59.7	2.7	79.4	4.6	84.0	2.4	85.1	1.3
5	Arterial	96.4		50.0		85.3		94.8		95.6	
	Venous	92.1	4.3	39.7	10.3	79.9	5.4	91.3	3.5	94.2	1.4
6	Arterial	95.0		65.7		91.0		92.6		91.5	
	Venous	90.7	4.3	56.2	9.5	83.7	7.3	85.9	6.7	86.4	5.1

Our findings clearly reflect such individual differences. In Subjects 1 and 4, for instance, insulin failed to exert any measurable effect on the peripheral rate of assimilation, and the A-V difference remained practically unchanged. This fact indicates the prompt response of the defense mechanism: the antagonistic factors appeared rapidly, and in a quantity that sufficed to put a brake on peripheral insulin action. In Subjects 3 and 5, on the other hand, the increased A-V difference at the first 30 minute interval showed that insulin action still was predominant over the antagonists and was effectively suppressed only with some delay. Still another variety of response was shown in Subject 2. In this instance the insulin-antagonistic mechanism reacted to hypoglycemia very efficiently, both in regard to the quantitative and chronologic aspects of the response; this is reflected in the fact that, despite the presence of insulin, the A-V difference not only failed to increase, but actually decreased. The resiliency of the process again manifested itself in the ensuing interval, when the blood sugar returned to (in fact had risen slightly above) the fasting level. At this stage, 90 minutes after the injection of insulin, the antagonistic mechanism promptly relaxed its activity and, in consequence, insulin was enabled to exert its peripheral action, as indicated by a substantial increase in the A-V difference. This contest, tug of forces, between insulin and its inhibitors explains why Cori and Cori (10) found, after the injection of insulin in fasted rabbits, considerable A-V differences over a period of 2 hours: Their rabbits were injected with nearly 5 units of insulin per kilo, more than 50 times our dose, so that the defense mechanism was unable to cope with the overwhelming action of the insulin, and in consequence peripheral insulin action prevailed and came to expression in greatly increased A-V differences.

The foregoing experiments, we realized, had distinct shortcomings. Although exploratory tests indicated that the lowest glycemic levels in healthy persons usually occur between 20 and 40 minutes after the injection of *small* doses of insulin, we probably missed this point in some of our six subjects. Likewise, we may have missed an increase in the A-V difference that may have taken place earlier than 30 minutes after the injection of insulin. The experiments would have undoubtedly been more informative if blood samples could have been drawn every 5 or 10 minutes; but this could have been accomplished only by using a micromethod for the analysis of capillary blood, inevitably at the expense of accuracy. As a compromise, in a second group of experiments we shortened the time intervals to 20 minutes. This second series differed from the first in two other respects. One difference was that we lowered the insulin dose to 3 units, with the expectation that individual variations might come to the fore even more clearly than with 5 units. Secondly, we performed on each of these subjects a second test, injecting 6 units of insulin, an increase of 100 per cent over the first dose. In this manner the difference between the action of two vastly different quantities of insulin could be observed, with the exclusion of the individual variations.

The results of such tests on five subjects are presented in Table II.

To facilitate comparison between the effects of 3 units and 6 units of insulin, only the venous blood sugar values are recorded, together with the A-V differences. (The arterial glycemic levels can be readily reconstructed from the two values.) These data supply definite information to the questions we have posed. In the first place, it is evident that 3 units of insulin did show up such individual differences which were obscured when a larger dose, like 5 or 6 units, was injected. Thus it may be noted that, after injection of 3 units, Subjects 10 and 11 were considerably less sensitive to insulin than the other three members of the group, a difference which was scarcely in evidence after the injection of 6 units. Another difference between individuals was the irregularity in the relationship between insulin dose and body weight. The blood sugar of Subject 10, for instance, who weighed 65 kilos, dropped only to 76.7 mg. per cent, while in Subject 11, who weighted 91 kilos, a greater decline was effected. Or compare Subjects 10 and 13, both of whom weighed about 65 kilos; yet in response to 3 units of insulin the lowest hypoglycemic levels were 63 and 27 mg. per cent, respectively. As a matter of fact, 3 units produced deeper hypoglycemias in Subjects 12, 13, and 14 than did 6 units in Subjects 10 and 11.

TABLE II

Paradoxical Effect of Increase in Insulin Dose upon Rate of Extrahepatic Glucose Assimilation

The venous blood sugar (B. S.) and arteriovenous differences (A-V) are expressed as mg. per 100 cc. of blood.

Subject No.	Insulin dose	Time interval after injection of insulin										Peripheral assimilation index
		Fasting		20 min.		40 min.		60 min.		120 min.		
		B. S.	A-V	B. S.	A-V	B. S.	A-V	B. S.	A-V	B. S.	A-V	
	units											
10	3	84.8	4.8	76.7	7.5	81.0	5.1	83.4	2.5	83.4	3.8	23.7
	6	87.5	2.1	46.7	11.4	39.7	5.4	70.5	0.8	89.9	5.4	25.1
11	3	84.8	7.0	63.2	3.8	73.7	7.0	82.9	5.4	82.4	6.2	29.4
	6	82.9	1.6	52.1	1.9	46.2	3.2	73.7	6.0	83.7	3.9	16.6
12	3	95.6	3.0	40.8	13.2	50.2	16.8	85.3	4.3	87.7	4.4	41.7
	6	96.1	2.7	35.1	8.1	47.0	4.3	78.8	7.6	94.8	4.3	27.0
13	3	92.1	5.1	27.0	14.1	53.2	3.2	58.8	19.0	86.8	10.4	51.8
	6	90.7	7.9	22.4	9.5	48.1	7.3	54.3	5.9	83.2	1.9	32.5
14	3	92.3	7.6	31.6	5.9	75.1	2.1	79.7	10.8	90.7	4.3	30.7
	6	87.8	4.0	25.1	2.6	55.6	3.0	62.6	13.5	92.3	6.3	29.4

The second question in these tests concerns the differences between the action of a smaller and a larger dose of insulin in the same individual. The data in Table II answer this question with the paradoxical fact that 6 units of insulin effected less increase in the peripheral glucose assimilation than 3 units. The difference between the effects of the two doses in some instances is obvious at a glance, if one compares the A-V differences. Thus in Subject 12 the maximum A-V difference after 3 units of insulin was 16.8 mg. per cent while after 6 units it was only 8.1 mg. per cent, and in Subject 13, for 3 units it was 19 mg. per cent, while for 6 units only 9.5 mg. per cent. Since, however, the A-V differences fluctuate as a result of the contest between insulin and its antagonists, the picture is not always quite so clear. A more adequate evaluation of peripheral insulin effects is possible by using as a basis of comparison the sum of the A-V differences that occurred at the several intervals after insulin injection. We designated this sum as the "peripheral assimilation index," and included it in Table II. The assimilation index, as may be noted, was in three instances significantly lower after 6 units than after 3 units of insulin, while in two subjects it remained virtually unchanged after doubling the dose.

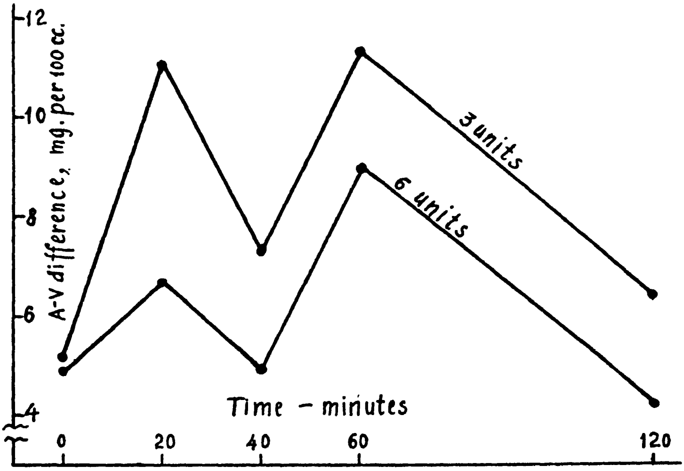

FIG. 1. Showing the paradoxical fact that in healthy man injection of 6 units of insulin promotes peripheral glucose assimilation to a lesser extent than 3 units.

To illustrate better the effects of the two doses of insulin, we present in Fig. 1 the composite A-V difference curves of Subjects 12, 13, and 14, and in Fig. 2 the corresponding venous blood sugar curves. The results for the insulin-"resistant" Subjects 10 and 11 are so different that they had to be set apart. These results cannot be understood without resorting to the concept that the changes in the glycemic levels are resultants of an interplay between insulin action and its antagonists. In Fig. 2 it may be noted that with 6 units of insulin hypoglycemia developed somewhat more precipitously and proceeded to a little lower level than after 3 units. The greater stress then exerted a greater stimulus on the defense mechanism, mobilized a larger supply of inhibitors, and, as a consequence, the larger insulin dose exerted less (and in two subjects no more) action on the peripheral glucose assimilation than the smaller one.

The two curves in Fig. 1 exhibit fluctuations which reflect the contest between insulin and its antagonists. The increase in A-V difference during the first 20 minutes indicates the predominance of insulin action; by this time, however, a large enough supply of antagonists had reached the field, with the consequence that insulin action was promptly depressed during the next interval, *i.e.* between 20 and 40 minutes after injection. This shift in the balance of forces was reflected in the shrinkage of the A-V difference, as well as in a simultaneous sharp upward swing (see Fig. 2) of the glycemic level. (The latter, of course, is a hepatic reaction of which we shall talk presently.) About 60 minutes after injection the blood sugar had risen to the neighborhood of the normal fasting level, a change which automatically lifted the stimulus which hypoglycemia exerted upon the insulin-antagonistic mechanism. As a result the insulin supply, that was

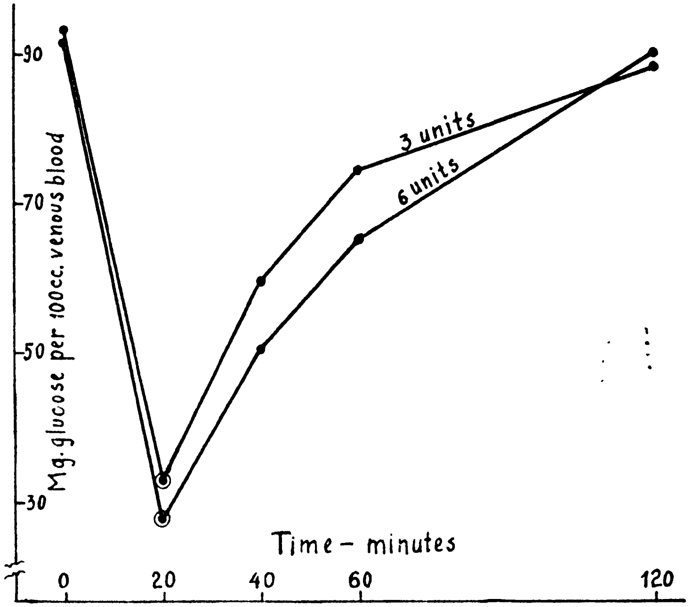

FIG. 2. Showing that in healthy man doubling of the insulin dose effects but slight difference in the blood sugar-time curve. After intravenous injection of either 3 or 6 units, the blood sugar begins to rise within 20 minutes after the onset of hypoglycemia, indicating the ascendency of insulin antagonistic action over insulin action.

still in circulation, once again was enabled to assert itself, as manifested by a second increase in the A-V difference (Fig. 1) (peripheral action), as well as in the change in the slope of the blood sugar curve (Fig. 2).

Hepatic Reactions to Hypoglycemia

In the defense reactions which combat hypoglycemia, the contribution of the peripheral (in the main, muscle) tissues is strictly negative; they put a brake on the downward progress of the glycemic level, but can neither stop it nor relieve it by returning to the blood glucose from their glycogen stores. This task is the burden of the liver. How does the liver accomplish this task? The generally accepted answer is that any increase in the blood sugar during postabsorptive states is due to an increase in the rate of hepatic glycogen breakdown. (It would be more appropriate, perhaps, to speak of glucose dissimilation, since glycogen breakdown implies only formation of glucose phosphate.) This is regarded as the sole factor that accounts, for instance, for the hyperglycemic effect of adrenalin and of anterior pituitary extracts, and should be responsible for the sharp upward swing of the glycemic level that follows in the wake of insulin hypoglycemia.

The validity of this concept seems to be taken for granted without question or doubt, although on closer scrutiny one finds that it would be difficult to support it with unequivocal experimental evidence. The most complete quantitative study in this field, done by Soskin and his colleagues (14), gives no information in this respect. These workers estimated the amount of glucose transported to the liver through the portal vein, designating this quantity as the "intake," and simultaneously determined the glucose carried off in the efferent vessels, calling it the "output." The meaning of these terms, if we do not misunderstand them, is properly expressed by Diagram I, in Fig. 3. It was convincingly demonstrated in these experiments that the glucose output from the liver becomes greater than the intake at the moment when the blood sugar declines below the normal fasting level. This shift, however, is no evidence of increased glucose dissimilation (glycogen breakdown), since the quantities determined show only the amounts of glucose entering and leaving the entire body of the liver, but represent neither assimilation ("intake"), nor dissimilation ("output") *by the liver cells.*

Diagram II, in Fig. 3, offers, we believe, a more faithful picture of the processes involved. The amount of glucose (A) that is transported to the liver does not simply flow through the liver cells, since these, like muscle and other tissue cells, are impermeable to free glucose. Only that fraction (a) enters the cells which is assimilated (via phosphorylation), while the unassimilated portion (b) by-passes the cells. This second fraction, the extracellular glucose, leaves the liver through its efferent vessels as a part of the "output" (E); to it is added the glucose derived from the continuous process of dissimilation (d), so that $E = b + d$. It is evident from this simple equation that E (the "output") can increase without an increase in d, that such a change can be produced by an increase in b (which in turn is a result of a decrease in a). A third possibility is that both b and d contribute to the changes in E. Which one of these three possible changes does actually take place? A glance at Diagram II (Fig. 3) reveals the virtually insurmountable difficulties in devising an approach for the experimental probing of the problem under reasonably physiological conditions. It is obvious that, while it is possible to measure A and E, these measurements fail to disclose whether an increase in E is due to an increase in d or a decrease in a (which entails an increase in b).

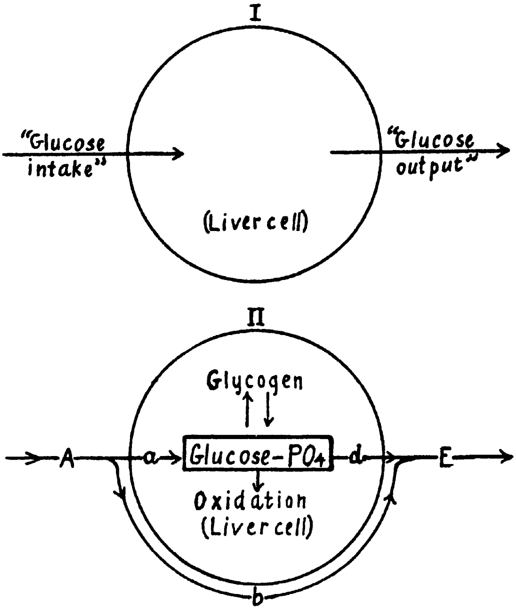

FIG. 3. The flow of glucose through the liver. Diagram I, an oversimplified erroneous presentation; Diagram II, a picture in harmony with current concepts concerning the carbohydrate metabolism of the liver. A is the amount of blood sugar transported to the liver; of this, fraction a is assimilated (*i.e.*, it enters the liver cell in the form of glucose phosphate) while fraction b, unassimilated, by-passes the cell and is carried off from the liver in the efferent vessels. To it is added d, the amount of glucose dissimilated by the liver cells.

In this situation it appears justified to postulate the assumption that the insulin antagonists, which are activated by hypoglycemia, depress the rate of glucose assimilation (decrease a) in the liver in the same manner as in the peripheral tissues. It is true that we have demonstrated this change only in the peripheral tissues. Since, however, glucose assimilation is catalyzed in both liver and muscle cells by identical enzyme systems, the inference is almost inescapable that factors which inhibit assimilation in muscle will elicit a similar response in the liver. One must not preclude, of course, the possibility that an increase in the rate of dissimilation may occur concurrently with the decrease in the rate of assimilation (*i.e.*, that an increase in both *a* and *d* may be set off simultaneously), especially in states of extreme stress and emergency.

Disparities between Insulin Supply and Insulin Action

The experiments thus far presented show that, unless hypoglycemia is completely excluded in studies of insulin action, one actually measures the balance of mutually opposing forces, the resultant of a contest between insulin and its antagonists, instead of true insulin action. It has come to our attention recently that Bouckaert and his associates (15) recognized this fact as early as 1929 and, with due regard to it, endeavored to maintain a near-to-normal glycemic level, by continual glucose infusion, while observing insulin action. In this country, Drury and Greely (16, 17), independently of the Belgian workers, devised a similar method, which enabled them to demonstrate that insulin acts much longer than indicated by the conventional procedure in which hypoglycemia is not only tolerated, but, in fact, the length of the hypoglycemic phase is regarded as the measure for the duration of insulin action.

The fallacy of this conventional approach is evident in Fig. 2, which shows that in healthy men the action of 6 units of insulin is just as short in duration (120 minutes) as is the action of 3 units. In the experiments of Norgaard and Thaysen (12), in which 12 units of insulin were administered to healthy men, the blood sugar returned to its fasting level within 100 minutes after injection, as short a time as after the injection of 3 units in our own experiments. This paradoxical phenomenon is undoubtedly due to the fact that in healthy persons hypoglycemia can mobilize enough insulin-antagonistic factors to counteract the action of from 3 to 12 units of insulin with nearly the same facility, in nearly the same brief period of time.

Experiments presented in Figs. 4, 5, and 6 clearly illustrate the causal connection between hypoglycemia and the frustration of insulin action. In Fig. 4, Curve I (which was taken over from Fig. 2) represents the response of healthy persons to 6 units of insulin, while Curve II shows the effect of the same dose in a hyperglycemic man (Subject A, who developed hyperglycemia following acute pancreatitis). As may be noted, in the hyperglycemic man insulin was still in action 3 hours after injection and, undoubtedly, proceeded beyond this period. It should be noted that in this instance the blood sugar was at no time even near a hypoglycemia level. In sharp contrast to this, in the healthy persons, in which hypoglycemia set in directly after the injection of insulin, the blood sugar began to rise sharply after 20 minutes. This was evidently the length of time necessary for the mobilization of an effective supply of insulin-antagonistic factors in the struggle against hypoglycemia.

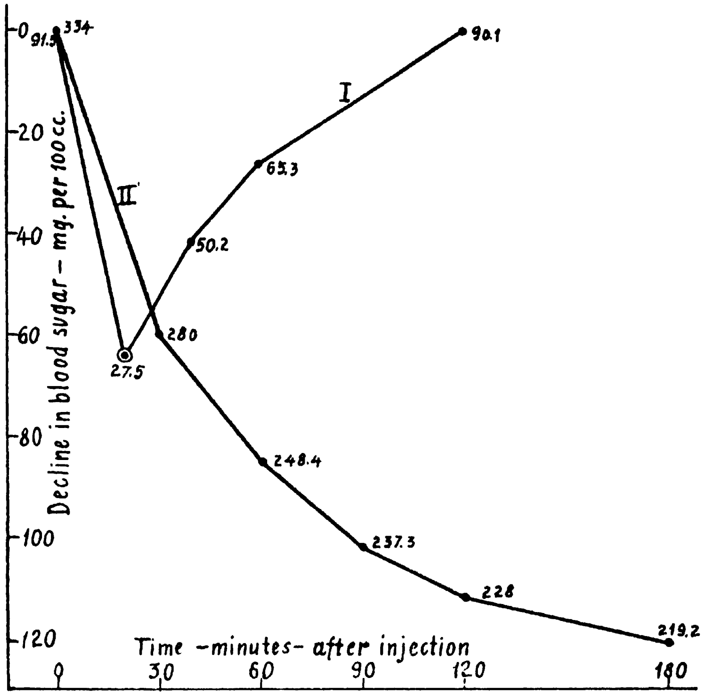

FIG. 4. Showing vast differences in the action of the same amount of intravenously injected insulin. Curve I represents the action of 6 units in three healthy men, Curve II the response to the same dose in a hyperglycemic man. The figures along the two curves represent the actual blood sugar values; the figures on the ordinate show the decline from the fasting level.

In Figs. 5 and 6, two more experiments are presented for additional illustration of the intimate connection between the occurrence of hypoglycemia and the duration of insulin action. The subjects in these experiments were diabetic persons who were managed by dietetic treatment alone (insulin therapy having been discontinued several years ago); for several years they had been aglycosuric and in as good physical condition as any healthy person. Each received 5 units of insulin intravenously in the morning, without breakfast. As may be seen, Subject B (Fig. 5) developed a mild hypoglycemia 30 minutes after injection; as a consequence, the blood sugar began to rise at that point. In Subject C (Fig. 6) hypoglycemia entered considerably later, only 90 minutes after injection; in consequence, it was at that time when the glycemic level began its

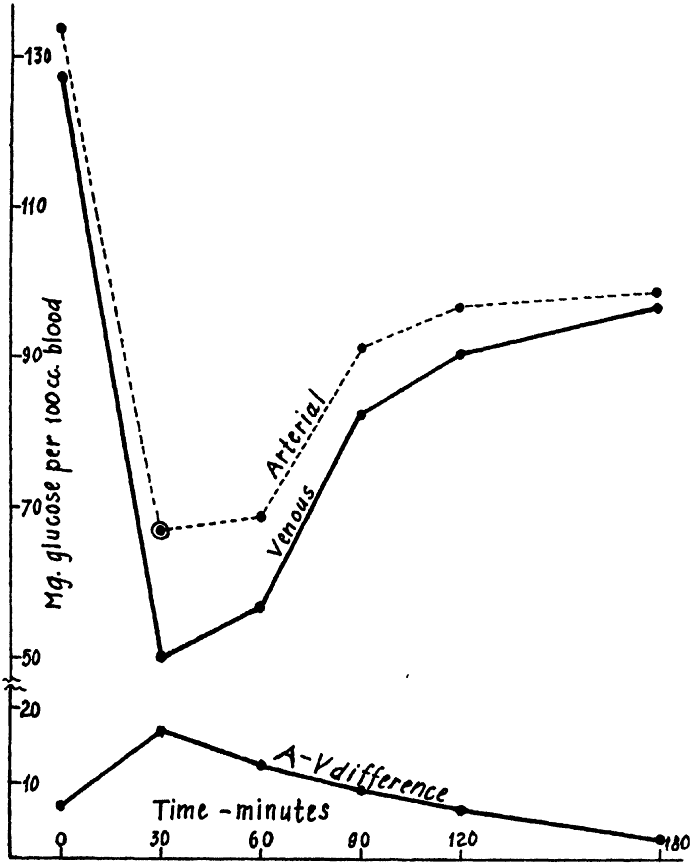

FIG. 5. Response to 5 units of intravenous insulin in a mildly diabetic man. The blood sugar began to rise in 30 minutes after injection, at the time when arterial hypoglycemia had set in (ringed dot). The A-V difference began to shrink at the same time. The vertical action of insulin was greater than in healthy persons because the road between the fasting blood sugar and the hypoglycemic level was longer.

upward swing. The fact that in Subject B the hypoglycemia was deeper and the ensuing upward turn sharper than in Subject C probably indicates that the insulin-antagonistic mechanism was more effectively excited in the former than in the latter. This interpretation is in full harmony with the changes in the A-V differences (shown in the curves in the lower parts of Figs. 5 and 6), as well as with the course of the blood sugar-time curves.

Hypoglycemia severely curtails not only the duration but also the intensity of insulin action, or, as it may be put, it causes a shrinkage not only of the horizontal, but also of the vertical dimension of the blood sugar-time curves. This fact is vividly illustrated in Fig. 4. It is evident from the two curves in this graph that the vertical action of insulin was abruptly stopped as soon as hypoglycemia activated the insulin-antagonistic mechanism sufficiently to turn the tide. In healthy persons this happened 20 minutes after the injection of insulin, when the blood sugar had decreased by 64 mg. per cent (a fall from 91.5 to 27.5 mg. per cent), whereas in the

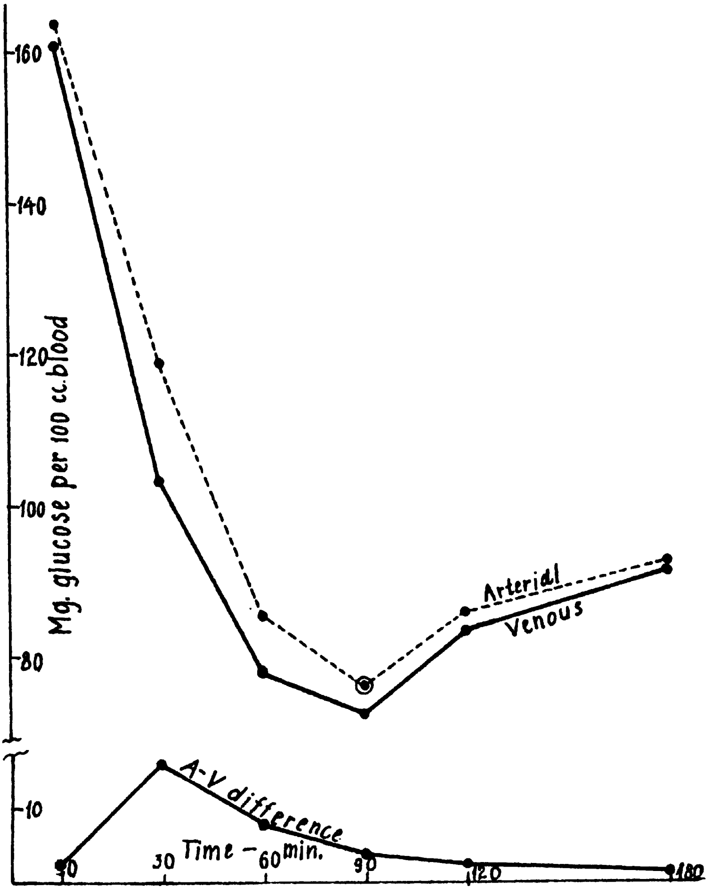

FIG. 6. An experiment like the one given in Fig. 5. In this mildly diabetic man 5 units of insulin intravenously produced *arterial* hypoglycemia in 90 minutes after injection (ringed dot), the point where the blood sugar began to rise.

hyperglycemic man the vertical action was as much as 118 mg. per cent (a fall from 341 to 223 mg. per cent), before insulin action was really over. This was possible simply because insulin action was introduced at a high glycemic level, and thus could proceed a long way before being impeded by hypoglycemia. The same relationship was evident in Subjects B and C. In the latter (Fig. 6) insulin action could depress the blood sugar by 89 mg. per cent before hypoglycemia prevented further downward progress, while in Subject B (Fig. 5), whose fasting blood sugar was lower, a hypoglycemic level was reached after a shorter vertical course, and hence the intensity of insulin action was confined to a smaller dimension.

A fourth experiment on a diabetic subject, recorded in Table III, shows all three features of insulin action that have thus far been considered. (1) Vertical insulin action (intensity) is greater if insulin is given in a hyperglycemic state than if injected at normal fasting levels. (2) The vertical action is stopped by hypoglycemia and, as a consequence, the horizontal action, too, is shortened. (3) The insulin antagonists, mobilized by hypoglycemia, inhibit glucose assimilation. This last fact is directly demonstrated by the changes of peripheral assimilation; as may be seen, the A-V difference increased to 40.5 mg. per cent as the result of insulin action. But the increase could be maintained only as long as hyperglycemia persisted; as soon as hypoglycemia appeared, the A-V difference promptly shrank to 3 mg. per cent and continued to decrease under the influence of the activated insulin antagonists.

TABLE III

Showing Self-Terminating Effect of Insulin Action (8 Units, Intravenously) if Hypoglycemia Is Allowed to Develop

Time after insulin	Glucose per 100 cc. of		A-V difference
	Arterial blood	Venous blood	
hrs.	*mg.*	*mg.*	*mg. per cent*
0	204.7	203.0	1.7
0.5	122.6	82.1	40.5
1	80.2	60.8	19.4
1.5	99.9	96.9	3.0
2	135.8	133.7	2.1
3	150.7	149.0	1.7

We have not mentioned thus far, but have not overlooked, the significance of changes in the rate of blood flow, which must be taken into consideration in the evaluation of A-V differences. It is obvious that changes in A-V differences do not correctly reflect changes in the rate of peripheral assimilation if the blood flow changes simultaneously. In our experiments, in which A-V differences decreased during hypoglycemia, we did not attempt to estimate the effect of possible changes in blood flow. This seemed unessential in view of the fact that hypoglycemia tends to decrease blood flow (14, 18), a change which implies an even greater decrease in the rate of assimilation than is indicated by the decrease in the A-V difference.

SUMMARY

The effects of intravenously administered insulin upon peripheral glucose assimilation were studied in healthy young persons in the postabsorptive state. It was found, as indicated by changes in the arteriovenous differences of blood sugar, that hypoglycemia, produced by insulin, entails a depression of the rate of peripheral assimilation. This response is ascribed to insulin-antagonistic endocrine factors (mainly to the pituitary-adrenal mechanism) which are activated by hypoglycemia. It is suggested that hypoglycemia leads to a depression of the rate of glucose assimilation in the liver as well as in the peripheral tissues. According to this concept the liver may contribute to the glucose content of the blood without any change in the rate of glucose dissimilation. Implications of the self-limiting action of insulin, which becomes effective as soon as the arterial blood sugar declines below the fasting level, are demonstrated and discussed.

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THE ABSORPTION SPECTRA AND EXTINCTION COEFFICIENTS OF MYOGLOBIN

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In a study of the quantitative variations of myoglobin in muscles, the need arose for reliable absorption spectra and extinction coefficients of derivatives of both myoglobin and hemoglobin in the visible range. Sufficient data on hemoglobin are available in the literature, but those for myoglobin (1, 2) are conflicting and suggest the need for further study. Therefore, it seemed advisable to publish the results of this study of the absorption spectra of myoglobin and its derivatives in the visible range. Since the maxima and minima of derivatives of myoglobin in the visible range are 2 to 10 mμ nearer the long wave-lengths than those of hemoglobin (1–4), it was thought of interest to study the absorption curves of myoglobin in the near infra-red and compare them with the curves of hemoglobin obtained by Horecker (5). Consequently, this paper presents absorption curves and molar extinction coefficients of horse heart myoglobin and several of its derivatives at wave-lengths from 1000 to 450 mμ. Studies were made of reduced (Mb) and oxidized, or met (MMb), forms and the derivatives, oxymyoglobin (MbO_2), carbonylmyoglobin (MbCO), and metcyanmyoglobin (MMbCN). Metmyoglobin (MMb) was studied at several pH values.

EXPERIMENTAL

The myoglobin used in these investigations was isolated by crystallization from horse hearts by a method described in an earlier paper (6). Material from five individual hearts was used. The purity of these five preparations was based on their iron content. If the iron in myoglobin is 0.323 per cent of the dry weight (6), purity of the preparations used in the present experiments was 96 per cent or better.

The stock solutions of myoglobin were diluted to concentrations appropriate for measurement at the wave-lengths to be studied. In the near infra-red the concentrations ranged from 0.15 to 0.40 $\times 10^{-3}$ M and in the visible from 0.03 to 0.07 $\times 10^{-3}$ M. After measurements were completed, the exact molarity of each solution was determined by converting the myoglobin to MMbCN by the addition of a trace of potassium ferricyanide and potassium cyanide. The density (d) was then measured at 540 mμ and the concentration determined by using 11.3×10^3, the molar extinction coefficient for MMbCN (7). The reliability of this method for determining the concentration of heme compounds as proposed by Drabkin (7) was tested by determination of the iron in six solutions of different concentrations of myoglobin. The concentrations obtained by the latter method agreed within 5 per cent of the values obtained by analysis as MMbCN.

For pH values from 6.0 to 8.0, Sørensen's phosphate buffers (8) in concentration of 0.5 M were added in proportions to make the final concentration 0.05 M. For pH values above 8.0, Sørensen's borate-HCl mixtures (8) and borate-NaOH mixtures (8) were used in final concentrations of 0.02 M.

Spectrophotometry—The measurements were made with a Beckman model DU spectrophotometer. The molar extinction coefficients were calculated by substitution in the Lambert-Beer law,

$$\log_{10} \frac{I_0}{I} = d = \epsilon cl$$

where I_0 = the intensity of incident light, I = the intensity of emergent light, d = the density as read from the spectrophotometer, ϵ = the molar extinction coefficient, c = the molar concentration, l = the length of the light path through the solution in cm., which in this instance equaled 1.00.

The nominal band width[1] ranged from 5.4 mμ at wave-length 1000 mμ to 0.6 at 450 mμ. At 580 and 540 mμ, two important wave-lengths, the nominal band widths were 1.2 and 1.0 mμ, respectively.

Preparation of Derivatives—Reduced myoglobin (Mb) was prepared by placing the properly diluted and buffered solution of MMb in a Thunberg tube modified in a manner similar to that of Theorell and de Duve (9). A few grains of sodium hydrosulfite were placed in the side arm. Prepurified nitrogen which had been passed over glowing copper was bubbled slowly through the solution for 10 minutes. Then the tube was tilted to dissolve the sodium hydrosulfite in the side arm and reduce the MMb. The slow bubbling of nitrogen was continued for another 3 minutes. The solution was transferred anaerobically to an absorption cell filled with nitrogen. The cell was stoppered and the measurements made.

Carbonylmyoglobin (MbCO) was prepared by similar treatment with the use of carbon monoxide from a generator instead of nitrogen. The solution was gassed for 10 minutes before and 3 minutes after reduction with sodium hydrosulfite.

Oxymyoglobin (MbO_2) had to be prepared by an indirect method.

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[1] The nominal band widths for the slit openings used were calculated from the graph in Bulletin 91-C, National Technical Laboratories, South Pasadena, California.

The direct oxygenation of reduced myoglobin resulted in rapid oxidation to MMb, possibly because, as with hemoglobin (10, 11), the reduced form is susceptible to oxidation. MbO_2 could be prepared, however, by first preparing MbCO and then displacing the CO with oxygen in a manner similar to that used by Theorell (12).

The apparatus for preparing MbO_2 consisted of a tonometer made from a 100 ml. round bottom flask onto the bottom of which was attached a straight glass nipple of dimensions to allow transfer of the solution to an absorption cell. About 1.8 ml. of a buffered solution (final pH 6.8) of MMb of appropriate concentration were put into the tonometer. The tonometer was then connected by rubber tubing and a glass-blower's swivel to a CO generator and rotated 40 to 50 R.P.M. with an electric motor. The solution was gassed with CO for 12 minutes. Then, while the CO continued to flow, the tonometer was opened and a few grains of sodium hydrosulfite were introduced. The MMb was immediately converted to MbCO. Rotating and gassing with CO were continued 3 more minutes. Then moisture-saturated oxygen was substituted for the CO.

TABLE I

Wave-Length of Maximum and Minimum Absorption of Derivatives of Horse Heart Myoglobin and Human Hemoglobin in Near Infra-Red

The values for hemoglobin were taken from Horecker's data (5), except for those of reduced horse hemoglobin.

Derivative	Position of maxima		Position of minima	
	Mb	Hb	Mb	Hb
	mμ	mμ	mμ	mμ
MbO_2	940-950	920	700	700
				680*
Mb	920	900	844	840
	760	760	740	731
		900†		840†
		756†		730†
MMbCN			800	800
Alkaline MMb	840‡	817.5§		

* Calf hemoglobin.

† Horse hemoglobin.

‡ pH 8.50 and 9.00.

§ pH 8.9.

In order to have the maximum amount of myoglobin present as MbO_2, the density of several solutions was measured at 582 mμ after different intervals of oxygen flow. These tests showed that the maximum absorption, therefore the maximum concentration of MbO_2, occurred after 3 to 4 minutes of oxygen flow. Fewer than 3 minutes of oxygen flow were not adequate to displace all the CO bound to myoglobin, and any MbCO that remained decreased the total density. More than 3 or 4 minutes of oxygen flow yielded lower densities because of oxidation to MMb. Consequently, the flow of oxygen was continued for 3 minutes in all preparations.

In the near infra-red region no special measures were taken to reduce the error caused by the oxidation of MbO_2 to MMb, except to make the readings necessary to explore the curve as rapidly as consistent with accuracy. In the visible region the error was diminished greatly by making but three measurements on a single solution. The first measurement was made at a maximum, or a minimum, and the second and third 2 mμ to either side of the first. This was carried out on three to five single solutions for each of the two maxima and the two minima in the visible region. The ϵ values for MbO_2 in Table IV were obtained in this manner.

Oxidized myoglobin (MMb) was prepared by diluting and buffering the stock solution. The hydrogen ion concentrations were measured, and in a few instances, when the pH was not sufficiently near that of the buffer added, solid dibasic potassium phosphate or sodium hydroxide was added to increase it to a value about 0.5 of a unit greater than that of the next lower solution.

The derivative metcyanmyoglobin (MMbCN) was prepared by the addition of solid cyanide to oxidized myoglobin.

RESULTS AND DISCUSSION

Absorption Spectra in Near Infra-Red Region—The absorption curves (Figs. 1 and 2) have the general shape of those of hemoglobin (5). As in the visible region, the maximum and minimum absorptions of myoglobin are located nearer the long wave-lengths than are those of hemoglobin (Table I). Reduced myoglobin (Mb) (Fig. 1) presents two maxima and two minima in the near infra-red. The difference between the wave-length of maximum absorption of Mb and Hb at 760 mμ is small (Table I). Horecker (5) gives 760 mμ as the maximum of human Hb. Since this is the position of the maximum for horse Mb, measurements were made of horse Hb. In each of five separate measurements (one sample of blood) on purified reduced hemoglobin, the maximum occurred between 755 and 757.5 mμ, or 2.5 to 5.0 mμ to the left of the maximum for Mb.

The molar extinction coefficients of MbO_2 (Table II) in the region of maximum absorption are about 20 per cent greater[2] than those for HbO_2 (5). This may be caused by the formation of acid MMb; however, calculations of the MMb that could be formed in the time (2 minutes) necessary to explore the curve between 1000 and 900 mμ indicate that MMb does not account for this increment over HbO_2.

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[2] The molar extinction coefficient for MbO_2 at 510 mμ (Table IV) is also greater than that of HbO_2 (5).

MbCO and MMbCN (Fig. 1), like these derivatives of hemoglobin, are noteworthy for their transparency to near infra-red radiation. MMb (Fig. 2) is equally noteworthy for the influence of the hydrogen ion concentration upon the character of its absorption in these wave-lengths. The absorption values of this compound are so affected by variations of pH that it was necessary to take the data in Fig. 2 from a single experiment. In many other experiments, the data agreed in general character with that presented, but the values at any particular wave-length do not duplicate each other because of small variations in pH. The results of Fig. 2 show the following: (1) at 1000 mμ the density of MMb decreases as the alkalinity increases between pH 7.0 and 9.8; (2) the density of MMb does not change as the acidity increases between pH 7.0 and 5.9 (myoglobin is unstable below pH 5.5); (3) an isosbestic point exists for all pH values of MMb at about 860 mμ; (4) an absorption band exists in MMb above pH 8.0 which increases in intensity and shifts toward the shorter wave-lengths as the pH increases.

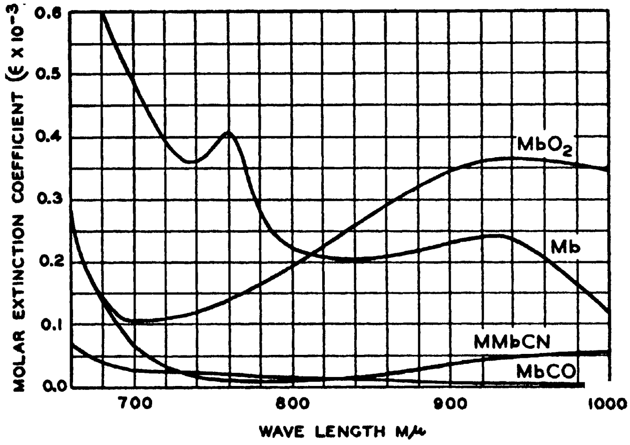

FIG. 1. Absorption curves for Mb, MbO_2, MbCO, and MMbCN in the near infra-red region.

Absorption Curves in Visible Wave-Lengths—The positions of the maximum and minimum absorptions of myoglobin and its derivatives in the visible range (Fig. 3, Table II) agree well with those in the literature (1–4), but there are differences between the extinction coefficients reported here and those reported elsewhere.

Two other extensive investigations have been made of the absorption curves of myoglobin (1, 2) made from crystalline material. Of these, Theorell (1) presents specific extinction coefficients, while Kiese and Kaeske (2) give only curves in which log I_0/I is plotted against wave-length. The values at the maxima and minima for four derivatives of myoglobin

TABLE II

Maximum and Minimum Molar Extinction Coefficients of Horse Heart Myoglobin and Its Derivatives between 450 and 1000 mμ

Derivative	No. of measurements	Wave-length	Molar extinction coefficients, $\epsilon \times 10^{-3}$		
			Average	High	Low
		mμ			
Mb	5	480	3.92	4.18	3.72
	4	555	12.92	13.09	12.72
	5	740	0.35	0.37	0.34
	5	760	0.40	0.42	0.39
	5	840	0.20	0.22	0.19
	5	920	0.24	0.25	0.23
MbO_2	3	510	5.5	5.6	5.5
	5	544	14.6	14.65	14.50
	4	564	8.5	8.51	8.42
	5	582	15.1	15.3	14.8
	4	700	0.10	0.11	0.10
	4	940	0.36	0.37	0.35
	4	950	0.36	0.37	0.35
MMbCN	5	510	6.87	7.07	6.43
		540	11.3*		
	7	780	0.04	0.08	0.01
MbCO	4	500	5.29	5.64	5.14
	6	540	14.85	15.00	14.62
	6	560	10.57	10.70	10.40
	5	577	12.95	13.15	12.72
Acid MMb,† visible	3	470	7.55	7.58	7.50
	3	500	9.85	9.90	9.82
	3	580	2.97	3.04	2.93
	3	630	3.71	3.66	3.77

* Drabkin's value (7).

† pH 6.00, 6.59, and 7.11.

studied in this investigation are compared with those of Theorell (1) and Kiese and Kaeske[3] (2) in Table III. The new values presented in this paper are intermediate to those from the literature, but for reasons presented,[3] no valid comparison can be made with the values given by Kiese and Kaeske. The new values agree substantially with those of Theorell. There are, however, disagreements which require comment. For example, the new ϵ values for MbO_2 (Table IV) are higher than those of Theorell. Possibly, since he oxygenated with air, his results were influenced by the myoglobin oxidizing to MMb. Neill and Hastings (10) and Brooks (11) have shown that hemoglobin oxidizes readily at the oxygen tensions of air and this may apply to myoglobin. The error introduced by the oxidation

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[3] As stated above, Kiese and Kaeske (2) show their results on hemoglobin and myoglobin as graphs in which log I_0/I is plotted against wave-length. They do not indicate the base of the logarithms, and it is impossible to tell which base they used by such methods as comparing their results with others established in the literature.

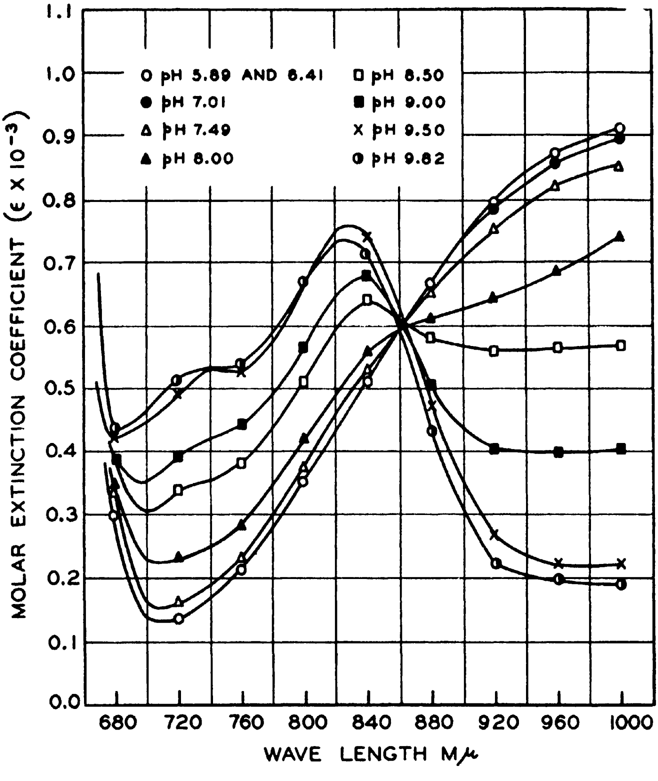

FIG. 2. Effect of pH on the absorption curves of MMb in the near infra-red region

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[3] For example, the molar extinction coefficient of HbO_2, a compound sufficiently stable to lend itself to accurate measurement, was found to be 15.0×10^3 at 540 mμ by Horecker (5) and 15.3×10^3 by Drabkin and Austin (13). If Kiese and Kaeske used $\log_{10}$ their concentration of hemoglobin (with 16,600 as the equivalent weight) makes $\epsilon = 19.5 \times 10^3$. If they used $\ln_e$, $\epsilon = 8.5 \times 10^3$. Neither of these values agrees sufficiently well with established values to give a clue for interpreting their results on myoglobin.

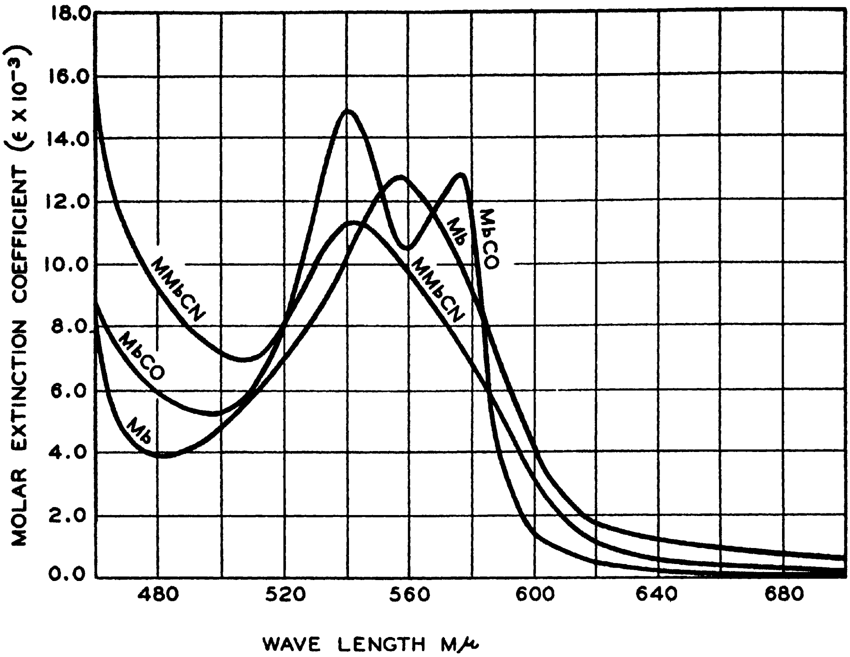

FIG. 3. Absorption curves for Mb, MbCO, and MMbCN in the visible region

TABLE III

Wave-Lengths of Maximum and Minimum Absorption of Myoglobin Derivatives and Their Molar Extinction Coefficients from This Paper and Literature

Derivative	Maxima						Minima					
	This paper		Theorell (1)		Kiese and Kaeske (2)*		This paper		Theorell (1)		Kiese and Kaeske (2)*	
	Wave-length	$\epsilon \times 10^{-3}$	Wave-length	$\epsilon \times 10^{-3}$	Wave-length	$\epsilon \times 10^{-3}$	Wave-length	$\epsilon \times 10^{-3}$	Wave-length	$\epsilon \times 10^{-3}$	Wave-length	$\epsilon \times 10^{-3}$
	mμ		mμ		mμ		mμ		mμ		mμ	
MbO_2	582	15.1	582	12.6	586	17.0	564	8.5	564	7.2	575	15.2
	544	14.6	542	12.3	547	17.5	510	5.5	500	4.5	512	8.2
Mb	555	12.9	555	11.2	555	29.8	480	3.9	480	3.5	480	9.3
MbCO	577	12.9	579	12.6	580	17.5	560	10.6	560	9.5	565	16.0
	540	14.8	540	12.3	540	19.2	500	5.3	500	4.8	495	8.3
			579	10.0†								
			540	12.0†								
MMb, pH 7.0	630	3.7	630	4.0	630	7.2‡	590	3.0	595	3.5		
	500	9.8	500	8.8	500	17.0	460	7.6	465	6.9	475	15.4‡

* The values in these columns are calculated on the basis that the authors used $\log_{10}$ and not ln, (see foot-note 3). The wave-lengths are approximations.

† The values for human MbCO are from Theorell and de Duve (9).

‡ The pH of these preparations is not given, but they were undoubtedly acid.

of MbO$_2$ to acid MMb in these experiments has been estimated to be no greater than 1.3 per cent. The new values for MbO$_2$ agree well with those found by Horecker (5) and Drabkin and Austin (13) for HbO$_2$.

Theorell (1) found the extinction at the α and β bands of horse MbCO to be about equal. Theorell and de Duve (9) found the extinction of the α band to be less than that of the β band in human myoglobin; however, their values for human myoglobin, transformed to ϵ, are lower than those for horse MbCO. If the extinction of MbCO in the α band is appreciably lower than in the β band it represents an outstanding difference between myoglobin and hemoglobin, because in HbCO the extinctions in the α and β bands are equal (5, 13). In these studies, measurements were made on six solutions of horse MbCO, and in each the difference (1.90×10^3) between the maximum ϵ values of the α and β bands (see Fig. 3) is substantiated. De Duve (14) has recently found this difference between the α and β bands in human MbCO in curves whose absorption values closely resemble those of horse MbCO reported here (Table II).

TABLE IV

Molar Extinction Coefficients of MbO$_2$ in Visible Region

The coefficients other than those necessary to indicate the wave-length of the maxima and minima are not shown because of the rapid oxidation of MbO$_2$ to MMb.

Wave-length	No. of readings	Molar extinction coefficients, $\epsilon \times 10^{-3}$		
		Average	High	Low
mμ				
584	4	14.8	15.1	14.7
582	5	15.1	15.3	14.8
580	4	14.7	15.1	14.3
566	3	8.6	8.6	8.5
564	4	8.5	8.5	8.4
562	3	8.6	8.6	8.5
546	5	14.4	14.5	14.4
544	7	14.6	14.7	14.5
542	5	14.3	14.4	14.2
514	3	5.6	5.62	5.53
512	3	5.5	5.56	5.50
510	3	5.5	5.56	5.50
508	3	5.6	5.60	5.52

The wave-length of the maximum absorption of Mb is very near, if not the same as that for Hb. In Table III it will be seen that the maximum for horse Mb occurs at 555 mμ according to Theorell (1), Kiese and Kaeske (2), and this study. The maxima for human (5) and dog (13) Hb also occur at 555 mμ. Density readings made in this study on purified horse Hb were found to be maximum between 553 and 556 mμ.

The absorption curves for MMb are given in Fig. 4. The maximum and minimum absorptions for acid MMb are given in Table II. Tabulated data for MMb at pH values above 7.0 are not presented because, as in the near infra-red, the absorptions are so affected by variations of pH that they are difficult to duplicate, and, consequently, curves are as useful as values

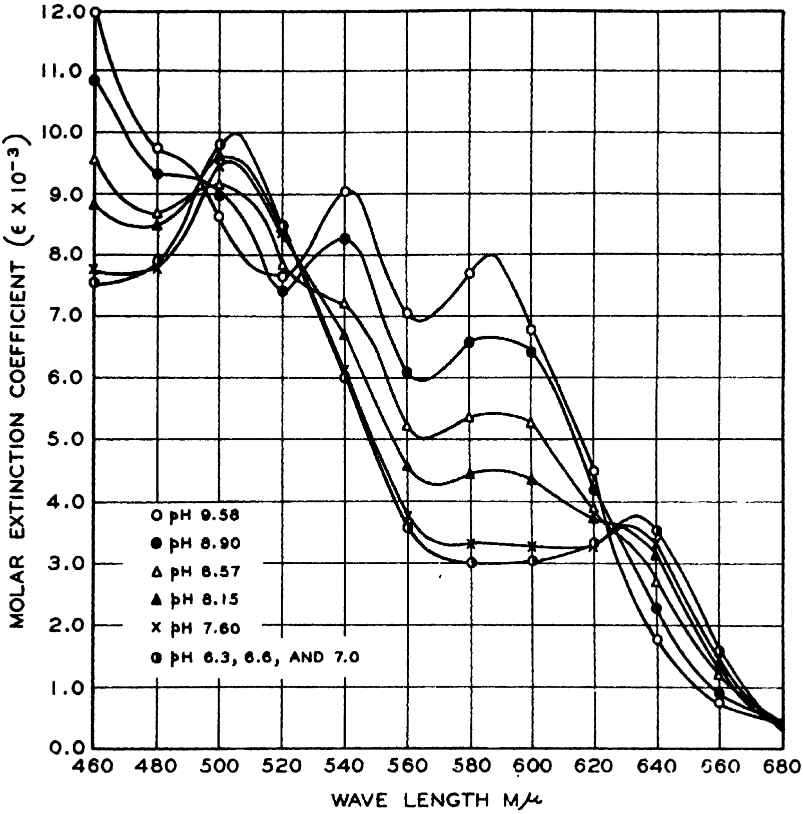

FIG. 4. Effect of pH on the absorption curves of MMb in the visible region

Below pH 7.0, MMb apparently exists as pure acid MMb. The family of curves in Fig. 4 resembles those of Haurowitz (15) and Austin and Drabkin (16) for methemoglobin (MHb). No effort was made to increase the pH to the alkalinity at which the myoglobin is 100 per cent alkaline MMb. The possibility of getting 100 per cent alkaline MMb is doubtful because of the formation, even though in small quantities, of alkaline hematin. Any alkaline hematin present would influence the densities sufficiently that duplication could not be obtained.

The absorptions of MMb are of the same magnitude, regardless of pH, at wave-lengths 628, 525, and 495 mμ (Fig. 4). Similar isosbestic points exist for MHb (15, 16), but in each instance the wave-lengths of equal absorption of MMb, like the wave-lengths of maximum absorption, are nearer the red (5 to 10 mμ) than for MHb.

SUMMARY

Absorption curves and molar extinction coefficients of horse heart myoglobin and several derivatives are presented for wave-lengths from 1000 to 450 mμ. In the near infra-red, the absorption curves resemble those of hemoglobin and, as in the visible range, the maxima are nearer the longer wave-lengths than those of hemoglobin. The absorption of metmyoglobin in the near infra-red is greatly influenced by pH values between 7.0 and 9.5, except at 860 mμ.

In the visible range the maximum absorptions of carbonylmyoglobin are not only nearer the longer wave-lengths than those of carbonylhemoglobin, but the absorption in one band is less than in the other, which is not true for carbonylhemoglobin. The maximum absorption of reduced myoglobin in the visible range appears to be at the same wave-length as that of hemoglobin. Oxymyoglobin has maximum absorption coefficients considerably higher than those previously reported and only sightly, if at all, less than those for hemoglobin. The absorption by metmyoglobin is greatly influenced by pH, and, since myoglobin oxidizes readily, this influence of pH is highly important in spectrophotometric studies.

The author appreciates the suggestions given by B. L. Horecker in this investigation.

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THE RÔLE OF PHOSPHATE IN THE METABOLISM OF PROPIONIBACTERIUM PENTOSACEUM

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Although it is commonly assumed that the fermentation of glucose and related substrates by propionic acid bacteria involves the phosphorylated intermediate compounds occurring in alcoholic fermentation, the evidence for this view is still incomplete. The first evidence for the occurrence of a phosphate ester in this type of fermentation was obtained by Virtanen (1) who observed a slow esterification of phosphate when glucose was acted upon by a preparation of dried bacteria. Virtanen and Karström (2) later isolated what they believed to be a hexose monophosphate from this system. No one has yet isolated either a hexose diphosphate or a triosephosphate from a propionic acid fermentation. The utilization of hexose monophosphate apparently has not been investigated, but there are several observations on the decomposition of fructose-1,6-diphosphate. Pett and Wynne (3) observed a conversion of magnesium hexose diphosphate to methylglyoxal under the influence of dried bacteria in the presence of toluene, and also demonstrated the presence of hexosediphosphatase, α- and β-glycerophosphatases, and a weak pyrophosphatase in their preparation. Werkman, Stone, and Wood (4) have shown that proliferating cells of *Propionibacterium pentosaceum* are able to use hexose diphosphate, 3-phosphoglycerate, and α-glycerophosphate as energy sources for growth in the absence of fluoride. The rates of decomposition of these substrates are much lower than for glucose. This could be due to the slow entry of the compounds into the living cells, or to the necessity for a preliminary hydrolysis, or possibly to other causes (5). The ultimate products are qualitatively the same as those formed from glucose. Werkman, Stone, and Wood (4) have shown the conversion of hexose diphosphate to phosphoglycerate by toluene-treated cell suspensions in the presence of fluoride and a suitable hydrogen acceptor. Phosphoglycerate can also be formed from glucose under similar conditions either in the presence or absence of a little hexose diphosphate (6). Pyruvate is the most effective hydrogen acceptor for the oxidation of glucose to phosphoglycerate (7). However, even without the addition of a hydrogen acceptor, a little phosphoglycerate is formed.

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This paper reports some observations on the rôle of phosphate in the decomposition of several substrates, mostly sugars and polyalcohols, by a dried preparation of *Propionibacterium pentosaceum*.

Materials and Methods

Dried cells of *Propionibacterium pentosaceum*, strain E.2.1.4, prepared by the method previously described by Barker and Lipmann (8) were used in all experiments. Several attempts to extract soluble enzymes from the dried bacteria were unsuccessful.

Two methods were used to study phosphate metabolism.

Method 1—To demonstrate the primary phosphorylation of a substrate by reaction with a high energy phosphate donor, the bacterial preparation was added to a system containing the phosphate acceptor to be studied, a phosphate donor such as ATP or phospho-enol-pyruvate, 0.005 M iodoacetate, 0.04 M to M/18 sodium fluoride, 0.1 M bicarbonate, and a carbon dioxide atmosphere. Iodoacetate inhibits oxidative processes and permits the accumulation of primary phosphate esters resulting from transphosphorylation reactions. Fluoride inhibits the decomposition of the phosphate esters. The transfer of phosphate from ATP results in the liberation of 1 mole of acid per mole of phosphate; in the presence of bicarbonate the rate of phosphate transfer can therefore be followed manometrically by observing the evolution of carbon dioxide. Reactions in this system were also followed by observing changes in properties of the phosphate compounds. The transferred phosphate was taken to be equal to the decrease in easily hydrolyzable phosphate (ΔP_7) minus the increase in inorganic phosphate ($\Delta P_{i.}$).

Method 2—To demonstrate the oxidative conversion of inorganic phosphate to phosphate esters, a system containing the oxidizable substrate, a suitable hydrogen acceptor such as pyruvate or fumarate (8), inorganic phosphate, 0.1 M bicarbonate, 0.04 M to M/18 sodium fluoride, and a carbon dioxide atmosphere was used. In this system the oxidation of the substrate was generally accompanied by the conversion of inorganic phosphate to a difficultly hydrolyzable ester of an organic acid such as phosphoglyceric acid. The rate of the process could therefore be followed manometrically by observing the liberation of carbon dioxide from the bicarbonate buffer. All experiments were done at 30°.

Inorganic phosphate was estimated by the method of Fiske and Subbarow (9), "true" inorganic phosphate by the method of Lipmann and Tuttle (10), phosphoglyceric acid by the polarimetric method of Meyerhof and Schulz (11), phosphopyruvic acid by the method of Lohmann and Meyerhof (12), reducing sugar by the ferricyanide method of Folin and Malmros (13), succinate by the manometric method of Krebs (14), pyruvate by the method of Lu (15), and lactate by the method of Barker and Summerson (16). The rate of hydrolysis of phosphate esters was determined by the method of Lohmann (17).

Results

Transphosphorylations from Adenosine Triphosphate (ATP) and Phosphopyruvate—By the use of Method 1, it was shown that the bacterial preparation was able to catalyze the transfer of phosphate from phosphopyruvate to glucose, arabinose, glycerol, and erythritol (Table I). No transfer was observed with sorbitol or mannitol. Essentially the same results were obtained in another experiment in which ATP was used in place of phosphopyruvate as a phosphate donor, except that sorbitol appeared to function to a slight extent as a phosphate acceptor.

TABLE I

Transphosphorylations from Phosphopyruvate

100 mg. of dried bacteria were suspended in 3 ml. of the solution described in Method 1. Incubation time, 120 minutes.

Substrates, 20 mg.	Phosphopyruvate added ($P_{pyr.}$)	Phosphate transferred ($\Delta P_{pyr.} - \Delta P_{i.}$)	Phosphate mineralized $\Delta P_{i.}$
	mg.	*mg.*	*mg.*
None	0.42	0.03	0.18
Glycerol	0.42	0.35	0.00
Erythritol	0.42	0.34	0.00
Sorbitol	0.42	0.00	0.25
Mannitol	0.42	0.00	0.22
Glucose	0.42	0.31	0.04
Arabinose	0.42	0.30	0.00

The relative rates of phosphate transfer from ATP to various substrates are illustrated in Fig. 1; the phosphate transfer is proportional to acidification and is measured manometrically as carbon dioxide liberated from the bicarbonate buffer. Fig. 1 shows that the rate of transfer decreases in the following order: glycerol, glucose, erythritol, and sorbitol. In other experiments arabinose was found to be phosphorylated at about the same rate as glucose.

It should be noted that one of the two labile phosphates of ATP is taken up rapidly, while the second follows at a much slower rate, if at all. This indicates that only the terminal phosphate of ATP transfers to the substrates of the glycero-, erythrito-, sorbito-, and pentokinases present in this preparation.

Coupling between Oxidation and Esterification of Inorganic Phosphate—

Method 2 was used to study the esterification of inorganic phosphate that accompanied the oxidation of various substrates.

Glycerol—Inorganic phosphate was rapidly utilized when glycerol and pyruvate were incubated in the presence of fluoride (Table II). The esterified phosphate was entirely in the form of a difficultly hydrolyzable ester which could be accounted for quantitatively as phosphoglyceric acid. The identity of the phosphoglyceric acid was established in a separate experiment by isolation of the crystalline barium salt which had a specific rotation of −684° in the presence of excess molybdate. The uptake of inorganic phosphate and the formation of phosphoglyceric acid were considerably inhibited by 0.005 M iodoacetate (Table II, Experiment 2).

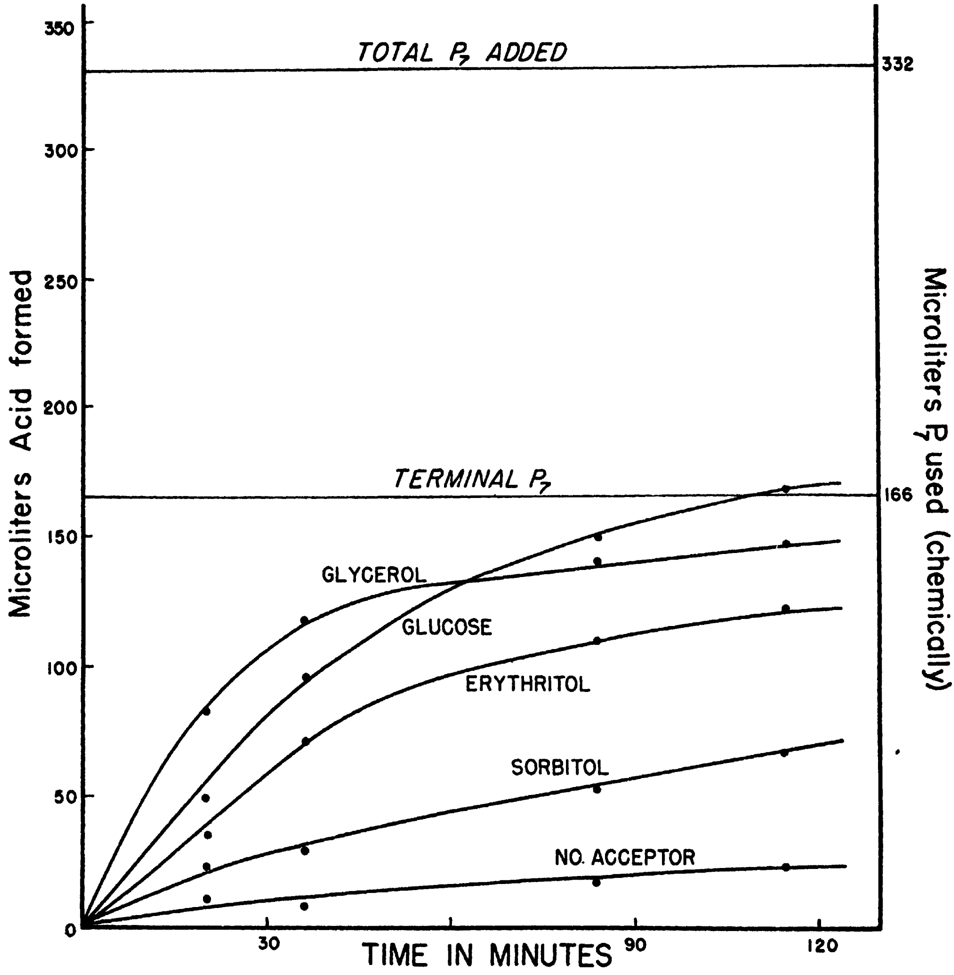

FIG. 1. The relative rates of phosphate transfer from ATP to various substrates

In other experiments it was shown that in this system pyruvate and fumarate are reduced to lactate and succinate, respectively. Data on the glycerol-succinate system are given in Table III. It can be seen that the quantity of succinate formed is approximately equivalent to the phosphate esterified.

TABLE II

Oxidative Phosphorylation of Glycerol and Erythritol Inhibition by Iodoacetate

70 mg. of dried bacteria were suspended in 3 ml. of the medium described in Method 2. Incubation time, 165 minutes. Quantities of substrates, pyruvate 100 μM, glycerol and erythritol 50 mg.

Experiment No.	Substrate	P_i.	P_7	ΔP_i.	ΔP (difficultly hydrolyzable)	ΔP (phosphoglyceric acid)*	CO_2 + acid
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*μl. per hr.*
1	None	0.47	0.11			0.16	49
	Pyruvate	0.37	0.175	−0.11	0.05		207
	" + glycerol	0.00	0.11	−0.47	0.47	0.69	397
	" + erythritol	0.21	0.09	−0.27	0.295	0.195	238
2 (0.005 M iodoacetate)	None	0.56	0.12				20
	Pyruvate	0.38	0.27	−0.18	0.02		227
	" + glycerol	0.34	0.25	−0.22	0.09		306

* Polarimetric method.

TABLE III

Oxidative Phosphorylation with Glycerol and Fumarate; Determination of Succinate

65 mg. of dried bacteria were suspended in 3 ml. of the medium described in Method 2. Incubation time, 130 minutes. Quantity of substrates, glycerol 50 mg., fumarate 100 μM.

Substrates	P_i.	ΔP_i.	Succinate formed	Acid formed
	mg.	*μl.*	*μl.*	*μl.*
None	0.415			
Glycerol	0.39	13	24	82
Fumarate	0.35	39	98	205
Glycerol + fumarate	0.00	292	233	580

Erythritol—The data in Tables II and IV show that erythritol is oxidized in the presence of pyruvate and fluoride with the uptake of inorganic phosphate and the formation of an approximately equivalent amount of a difficultly hydrolyzable ester. The latter has not been identified, but it cannot be phosphoglyceric acid, judging from the results of the polarimetric estimation of this compound. No extra carbon dioxide was formed when erythritol was added to pyruvate, but extra acid was formed, equivalent to about 0.5 mole per mole of phosphate esterified.

TABLE IV

Oxidative Phosphorylation with Erythritol and Pyruvate; Determination of Phosphoglyceric Acid

140 mg. of dried bacteria were suspended in 3 ml. of the solution described in Method 2. Incubation time, 250 minutes. Quantity of substrates, erythritol 80 mg., pyruvate 250 μM.

Substrates	P_i.	P_7	ΔP_i.	ΔP (difficultly hydrolyzable)	P (phosphoglyceric acid)*	CO_2 + acid
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*μl.*
None	1.23	0.13			0.21	192
Erythritol	1.28	0.13	0	0	0.26	146
Pyruvate	0.81	0.41	−0.42	0.14	0.30	2405
" + erythritol	0.30	0.10	−0.93	0.96	0.39	2700

* Polarimetric method.

TABLE V

Oxidative Phosphorylation with Sorbitol and Mannitol

150 mg. of dried bacteria were suspended in 3 ml. of the solution described in Method 2. Incubation time, 540 minutes (Experiment 1) and 120 minutes (Experiment 2). Quantities of substrates, pyruvate 250 μM, sorbitol 80 mg., mannitol 50 mg.

Experiment No.	Substrates	P_i.	P_7	ΔP_i.	ΔP (difficultly hydrolyzable)	P (phosphoglyceric acid)*
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
1	None	2.76	0.16			0.09
	Pyruvate	2.10	0.52	−0.66	0.30	0.35
	Sorbitol	2.73	0.17	−0.03	0.0	0.07
	Pyruvate + sorbitol	1.61	0.35	−1.15	0.95	0.47
2	None	0.77	0.00			
	Pyruvate	0.49	0.19	−0.28	0.095	
	" + mannitol	0.39	0.16	−0.38	0.23	

* Polarimetric method on lead precipitate.

Sorbitol—Sorbitol alone was not metabolized to an appreciable extent, but when pyruvate was also present the esterification of inorganic phosphate was about twice as great as with pyruvate alone (Table V). Most of the ester formed from sorbitol was difficultly hydrolyzable, but only a part of it, not more than half and probably much less, was phosphoglyceric acid. The remaining part was not identified. The available data on the formation of acid in the oxidation of sorbitol are not entirely consistent, but indicate a small formation of acid. No carbon dioxide was produced under the conditions of these experiments.

Mannitol—This compound behaved much like sorbitol (Table V). There was a slow uptake of inorganic phosphate with the formation of a difficultly hydrolyzable ester that was not phosphoglyceric acid. Acid production was much smaller than with glycerol.

Inositol—In the presence of pyruvate as an oxidant, inositol was metabolized with the formation of acid and a reducing compound, but there was no significant uptake of inorganic phosphate (Table VI). The increase in reducing value calculated as glucose (15.8 µM) was approximately equivalent to the extra acid (13.0 µM). The reducing value was not appreciably altered by 7 minutes hydrolysis at 100° in N HCl, but after such treatment a compound was formed which reacted with the Fiske-Subbarow molybdate reagent to give a green color that interfered with phosphate determinations. Before hydrolysis this effect was not produced.

TABLE VI

Oxidative Phosphorylation with Inositol and Pyruvate

100 mg. of dried bacteria were suspended in 3 ml. of the solution described in Method 2. Incubation time, 180 minutes. Quantity of substrates, inositol 50 mg., pyruvate 150 µM.

Substrates	P_i.	P_7	"Glucose"	CO_2 + acid
	mg.	*mg.*	*mg.*	*µl.*
None	0.75	0.09	0.98	183
Inositol	0.76	0.08	1.00	190
Pyruvate	0.44	0.32	1.12	809
Inositol + pyruvate	0.40	*	3.96	1100

* After 7 minutes acid hydrolysis this sample gave a greenish color with molybdate alone that interfered with the phosphate determination.

Glucose—Glucose alone was metabolized slowly in the presence of fluoride, but on the addition of pyruvate or fumarate as an oxidant, there was a fairly rapid formation of acid and carbon dioxide and an uptake of inorganic phosphate. Typical data are given in Table VII. The molar ratio of phosphate esterified to glucose decomposed, corrected for the control with fumarate alone, varied in different experiments from 1.1 to 1.7, averaging about 1.5. Since there is evidence for a slow hydrolysis of the phosphate esters, it seems likely that the bacterial preparations are capable of esterifying a maximum of 2 moles of phosphate per mole of glucose.

The nature of the phosphate ester was investigated in some detail. Triose phosphate and phosphopyruvate were shown to be absent; no inorganic phosphate was liberated by alkaline hydrolysis or by oxidation with iodine in alkaline solution. Most of the ester withstood hydrolysis with N HCl for 2 hours, suggesting the presence of phosphoglyceric acid. In a larger scale experiment in which glucose and pyruvate were incubated for 7 hours at 30° in the presence of M/18 fluoride and 0.025 M phosphate, phosphoglyceric acid was isolated and identified by its optical rotation ($[\alpha]_D = -700°$) and the phosphorus content of the barium salt (found 8.35 per cent; theoretical 8.53 per cent). The twice recrystallized product represented 54.5 per cent of the total phosphate taken up. Application of the polarimetric method of Meyerhof and Schulz (11) indicated that all the ester phosphate in the reaction mixture was phosphoglyceric acid.

TABLE VII

Oxidative Phosphorylation with Glucose, Arabinose, and Fumarate

150 mg. of dried cells were suspended in 3.5 ml. of a solution containing 0.1 M $NaHCO_3$, M/18 NaF, and 0.025 M K_2HPO_4. Gas phase, CO_2. Quantities of substrates, fumarate 100 µM, glucose and arabinose 40 µM. Incubation time, 385 minutes.

Substrate	Sugar consumed	CO_2 + acid	ΔP_i.	Volatile acid	$\frac{\Delta P_i.}{\Delta \text{ sugar}}$
	µl.	µl.	µl.	µl.	
Fumarate		304	−111	708	
" + arabinose (total)	609	1014	−669	1030	
" + " (corrected)*	609	710	−558	322	0.92
Fumarate + glucose (total)	666	1189	−836	960	
" + " (corrected)*	666	882	−725	252	1.09

* Corrected for control with fumarate alone.

Arabinose—This compound behaved very much like glucose. The data of Table VII show that the oxidation of arabinose resulted in the formation of acid and carbon dioxide and the esterification of phosphate to the extent of 0.92 mole per mole of arabinose decomposed. In several experiments, this ratio, corrected for the control without arabinose, varied from 0.92 to 1.25, the average being about 1.05. This is appreciably smaller than the ratio obtained with glucose. The phosphate ester was shown to consist entirely of phosphoglyceric acid by the method of Meyerhof and Schulz. Almost pure barium phosphoglycerate was isolated in 36.5 per cent yield ($[\alpha]_D = -689°$; P 8.22 per cent). There was some formation of volatile acid from arabinose in spite of the presence of fluoride, the yield in two experiments being approximately 0.5 mole per mole of arabinose. About the same yield of volatile acid was also obtained from glucose (Table VII).

Pyruvate—The fermentation of pyruvate in the presence of fluoride was accompanied by a small esterification of phosphate (Tables IV and VI). The ratio of phosphate esterified to pyruvate decomposed varied from 0.09 to 0.33. The highest value was obtained with 0.1 M sodium fluoride. In the absence of fluoride no inorganic phosphate disappeared.

Most of the inorganic phosphate utilized could be accounted for by an increase in easily hydrolyzable phosphate. This ester was shown to be precipitable by barium ion at pH 5 but was not further characterized. The ester is probably not ATP, since the quantity formed in some experiments appeared to be greater than could be accounted for by the adenylic acid content of the system. Possibly the compound is an inorganic pyrophosphate. Acetyl phosphate and phosphopyruvate could not be detected among the products of pyruvate decomposition.

DISCUSSION

The experimental results support the belief that propionic acid bacteria utilize phosphate compounds in much the same way as do other organisms that have been more thoroughly studied, and they also provide some specific information concerning the breakdown of sugars and polyalcohols.

The data show that glucose, arabinose, glycerol, and erythritol are rapidly phosphorylated by a reaction with ATP. Phosphopyruvate can also serve as the phosphate donor, but it is probable that this compound acts through the adenylic acid system. In contrast with the above substrates, mannitol and sorbitol are phosphorylated very slowly or not at all by reaction with ATP, although these compounds are readily fermented.

With glucose as a phosphate acceptor considerably more than 1 mole of phosphate was transferred per mole of glucose, indicating a formation of hexose diphosphate. The latter compound is probably converted to triose phosphate and then oxidized. The experiments on the oxidation of glucose in the presence of fluoride with pyruvate or fumarate as a hydrogen acceptor showed that between 1.5 and 2 moles of phosphate were esterified per mole of glucose and that all of this phosphate was in the form of phosphoglyceric acid.

With arabinose it was of interest to find out whether a mono- or a diphosphate derivative was formed. Unfortunately the data are not conclusive on this point, but they indicate that approximately 1 mole of phosphate is esterified per mole of arabinose oxidized. The ester was shown to be entirely phosphoglyceric acid. These results suggest that arabinose is converted to a pentose monophosphate which is split into a triose phosphate and an unphosphorylated C_2 fragment.

The transphosphorylation experiments indicate that glycerol is converted to a monophosphate ester. With fumarate as a hydrogen acceptor, the glycerophosphate was evidently oxidized first to triose phosphate and then to phosphoglyceric acid, which accumulated almost quantitatively in the presence of fluoride.

Like glycerol, erythritol reacts with ATP to give what is probably a monophosphate. Since the oxidation of erythritol in the presence of fluoride results in the formation of acid and the esterification of inorganic phosphate, it is likely that the following reactions occur.

$$\begin{aligned}&\mathrm{CH_2OHCHOHCHOHCH_2OH} + \sim ph \rightarrow \mathrm{CH_2O}ph\mathrm{CHOHCHOHCH_2OH} \xrightarrow{-2\mathrm{H}}\\ &\mathrm{CH_2O}ph\mathrm{CHOHCHOHCHO} \xrightarrow[]{\substack{-2\mathrm{H}\\ +\mathrm{HPO_4^=}}} \mathrm{CH_2O}ph\mathrm{CHOHCHOHCOO}ph \rightarrow\\ &\mathrm{CH_2O}ph\mathrm{CHOHCHOHCOOH} + \sim ph\end{aligned}$$

This scheme is further supported by the observation that neither phosphoglyceric acid nor carbon dioxide is formed in the oxidation of erythritol. We do not have any information on the further decomposition of the postulated C_4 acid in a normal fermentation.

Sorbitol is apparently not directly phosphorylated. The data are consistent with the view that sorbitol is first oxidized to a sugar which is phosphorylated and then oxidized with the generation of an energy-rich phosphate bond. The ester which accumulates is difficultly hydrolyzable, but cannot be phosphoglyceric acid. Mannitol seems to behave very much like sorbitol.

There is considerable evidence that propionic acid bacteria form acetate by an oxidative decarboxylation of pyruvate (18). Since the oxidation of pyruvate by *Lactobacillus delbrueckii* was found to involve the intermediate formation of acetyl phosphate (19), it was thought likely that this compound would also be formed in pyruvate oxidation by propionic acid bacteria. However, all attempts to detect acetyl phosphate in our preparations were unsuccessful. If acetyl phosphate is actually formed, it must be rapidly decomposed. Most of the inorganic phosphate taken up is converted to an easily hydrolyzable compound that has some of the properties of inorganic pyrophosphate, although an organic pyrophosphate is not definitely excluded.

Unlike the other fermentable substrates examined, inositol was neither phosphorylated directly by ATP nor oxidized with an uptake of inorganic phosphate. The oxidation of inositol in the presence of fluoride results in the formation of an acid and a reducing compound in approximately equivalent amounts. If the acidic and reducing properties belong to the same compound, the product of inositol oxidation may be a uronic acid or a somewhat similar compound. In this connection it may be noted that Kluyver and Boezaardt (20) observed the formation of inosose, a reducing cyclose, as a result of the oxidation of inositol by *Acetobacter suboxydans*. The propionic acid bacteria probably cause a further oxidation with opening of the ring.

SUMMARY

Transphosphorylations and the oxidative formation of phosphate esters have been studied with dried preparations of *Propionibacterium pentosaceum* with several substrates including glucose, arabinose, glycerol, erythritol, mannitol, sorbitol, inositol, and pyruvate. The results show the importance of phosphate in the metabolism of propionic acid bacteria and provide some information concerning the manner in which the substrates are decomposed.

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POLYMERIZATION OF 6-HYDROXYINDOLES AND ITS RELATION TO THE FORMATION OF MELANIN

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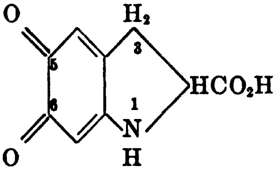
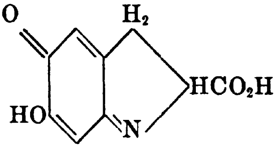
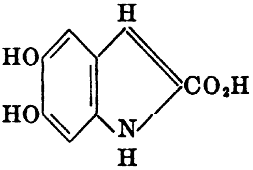
Melanins are probably the most widely distributed pigments in the animal kingdom. In the laboratory one can be formed by oxidative processes from 3,4-dihydroxyphenylalanine. On excellent grounds (1) an intermediate product (2) is judged to be an *o*-quinone indoline, I, or one of its tautomers, for example II or II′. Recent spectroscopic measurements (3) clearly favor I.



(I)



(II)

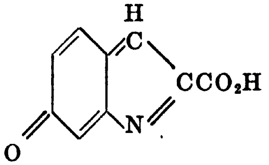


(II′)

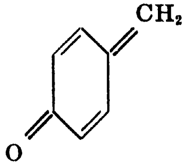
Subsequent oxidation of this intermediate yields a melanin. The course of this change is not understood, although it seems clear (1–3) that tautomeric changes occur to I so that structures of the type of II or II′ form.

Some light might be thrown on the subject if the behavior of monohydroxyindoles was known. 5-Hydroxyindole (4) is stable unless it happens to be formed in acid solution (5), where it can suffer the same type of vinyl polymerization which indole and skatole (6) undergo. 6-Hydroxyindole has not yet been prepared. Good reasons exist for supposing that such a compound would have unusual polymerizing activity. For instance, the 3 position of indole is normally very active as seen in the Mannich reaction, certain condensation reactions, and the behavior with Grignard reagents. Mild oxidation, such as is used for changing hydroquinones to quinones, should convert a 6-hydroxyindole to a quinomethane, III. Quinomethanes contain an unsymmetrically substituted vinyl system and as a class are highly susceptible to polymerization. The simplest quinomethane, IV, has never been prepared, but if two phenyl groups are present, as in the well known fuchsone, V, there is sufficient stability to permit isolation. The *as*-diphenylquinodimethane, $(C_6H_5)_2C{=}C_6H_4{=}CH_2$, cannot be isolated (7) because of its great tendency to polymerize. While higher molecular weight analogues, such as dinaphthylquinodimethane,

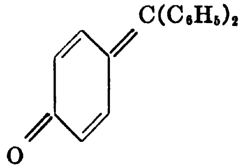
$(C_{10}H_7)_2C=C_6H_4=CH_2$, can be isolated, they polymerize readily. In general a vinyl group unsymmetrically substituted and with 1 carbon part of the ring seems highly disposed toward polymerization.



(III)

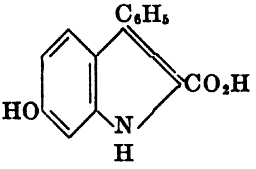


(IV)

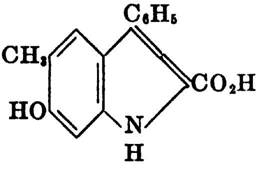


(V)

These facts led us to study compounds (*a*) in which an *o*-quinone might not, or could not, form and (*b*) in which a single phenyl group is at the 3 position in order to facilitate the isolation of what might otherwise prove to be an exceedingly unstable monomer. Such a phenyl group is often used[1] in organic problems because of its stabilizing nature and, in the present instance, was expected to impart desirable characteristics. The two compounds selected were 2-carboxy-6-hydroxy-3-phenylindole (VI) and its 5-methyl homologue (VII), which, apart from the features noted, are very similar to the indole, II, from 3,4-dihydroxyphenylalanine.



(VI)



(VII)

Compound VI or its methyl ester could be isolated only in impure form although the 6-benzyloxy-3-phenylindole-2-carboxylate that immediately preceded VI was quite stable. The product obtained was always low in carbon, probably because of oxygen uptake at position 5. Causse's (8) test with antimony trifluoride for *o*-diphenols was positive. The 5 position would be active because of the combined influence of the 6-hydroxy and the amino group. When the 5 position was blocked, as was done later in the preparation of VII, no oxygen was taken up. Hence the desired product, VI, was probably contaminated with the 5,6-dihydroxy compound (VIII).

Isolation of pure VII proved feasible. Its oxidation with potassium chlorate-vanadium pentoxide or with ferric chloride, both mild reagents

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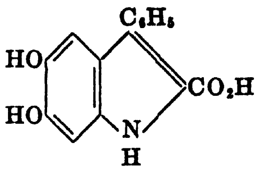
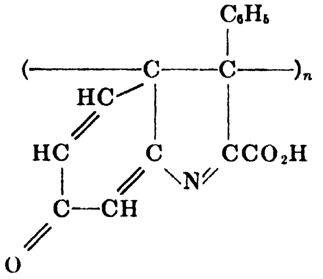
[1] For example, phenyl groups stabilize carbonium ions so that the isolation of the stable carbonium triarylcarbonium perchlorate is possible. It also slows down the tautomeric process of nitromethane so that the change can be observed. It is responsible for the unreactivity of chlorine in chlorobenzene.



(VIII)

suitable for converting hydroquinones to quinones, yielded dark amorphous polymers, insoluble in common organic solvents and in dilute alkali. It is therefore clear that the 5 position is unnecessary for conversion to a melanin-like product. Polymerization takes place with great ease, even when the course of oxidation is restricted to the quinomethane path and a phenyl group is present to impart stability.



(IX)

While a vinyl function appears to be present in the hypothetical primary oxidation product from VII and can cause polymerization, the actual course is obscured by one or more secondary reactions. A vinyl type of polymerization might give a structure, IX, for which the empirical composition would be 2 hydrogen atoms less than for the monomer. But the products from polymerization showed higher percentages of carbon with loss in acidity. Carbon dioxide could not have been lost, because that would require a very large increase in percentage of carbon. Furthermore the ratio of carbon to nitrogen remained approximately constant at 16:1, rather than dropping to 15:1. The general change in composition indicates a condensation and the simplest one would be loss of water, the OH coming from the carboxyl group and the H from the NH of another unoxidized molecule. The over-all oxidation-condensation process would then be expressed by the equation

$$2C_{16}H_{11}O_3N—2H—H_2O=C_{32}H_{18}O_5N_2$$

The composition of the polymers produced in acetic acid was not far from this value. See Table I.

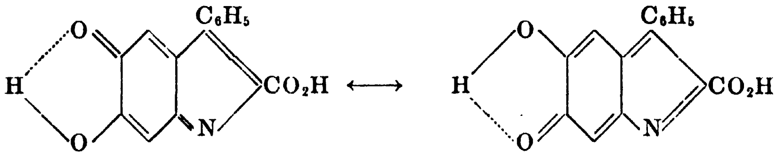
The argument that the carboxyl group participates in an ordinary condensation as a secondary reaction during the polymerization seems to be sustained from the results in alcohol in which esterification could occur. In this case the ratio of atoms of carbon to nitrogen would approach 18 the more successfully esterification competes with the alternative condensations. The data in Table I are consistent with this view.

TABLE I

Comparison of Atoms in Polymers from 6-Hydroxy-5-methyl-3-phenylindole-2-carboxylic Acid

	Ratio of atoms in polymer			
	C	N	H	O
Monomer	16	1	13	3
Polymer (theory)	16	1	11	3
" (found in HOAc with $KClO_3 \cdot V_2O_5$)	16.05	1	11.3	2.46
With $FeCl_3$	16.4	1	12.9	2.1
Polymer less H_2O; theory for oxidation-condensation	16	1	9	2.5
Polymer (found in EtOH with $KClO_3 \cdot V_2O_5$)	17.20	1	12.55	2.69
With $FeCl_3$	17.3	1	14.7	2.74
Polymer; theory for oxidation-esterification	18	1	15	3

These results do not of course prove that the polymerization of a compound, such as II′ or VIII, which has the hydroxyl group at C_5, must take place through the quinomethane path, but it is conceivable that polymerization does occur that way and that the hydroxyl group at C_5 activates, but does not directly participate in, the process. Attention should be drawn to the fact that, if oxidation had taken place to give a quinoimine, X, as the initial step, the product would still be equivalent, in degree of oxidation, to a quinomethane, XI, since the two structures can be pictured as contributing to resonance in a hybrid, as indicated by the formulas. The second form could then be the one that would yield the polymer.

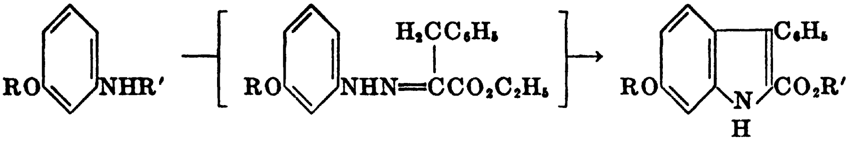


(X) (XI)

The methods of syntheses of the two compounds are outlined in Diagrams 1 and 2. In general, each preparation involved the application of a Japp-Klingemann condensation to an appropriate intermediate in which the phenolic hydroxyl was protected by a benzyl group. The latter was then removed by aluminum chloride.

An attempt was made to prepare 1-methyl-3-phenyl-6-hydroxyindole in which the tendency for the 5 position to oxidize might be lessened. The method chosen necessitated the formation of the unsymmetrical hydrazine, *m*-$C_6H_5CH_2OC_6H_4N(CH_3)NH_2$, which was to be used for the preparation of a hydrazone and a Fischer indole synthesis. The scheme proceeded satisfactorily through the preparation of the nitroso compound, *m*-$C_6H_5CH_2OC_6H_4N(CH_3)NO$, but met failure in the reduction to the hydrazine.

Diagram 1. Method of Preparation of Compound VI



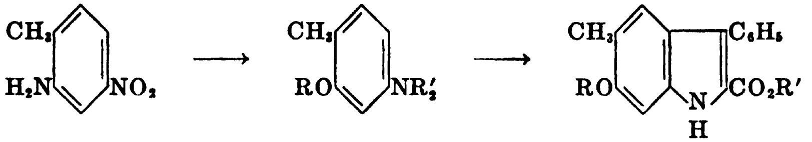
(XII to XV)

(XVI and VI)

XII, R = H, R′ = H
XIII, " = " " = $COCH_3$
XIV, " = $C_6H_5CH_2$, R′ = $COCH_3$

XV, R = $C_6H_5CH_2$, R′ = H
XVI, " = " " = C_2H_5
VI, " = H, R′ = H

Diagram 2. Method for Preparation of Compound VII



(XVII)

(XVIII to XX)

(XXI and VII)

XVIII, R = H, R′ = O
XIX, " = $C_6H_5CH_2$, R′ = O
XX, " = " " = H

XXI, R = $C_6H_5CH_2$, R′ = C_2H_5
VII, " = H, R′ = H

An attempt was made also to prepare VI by way of the *O*-methyl instead of the *O*-benzyl ether. The experiments were successful until the last stage of cleavage, in which extensive polymerization took place to give a black solid.

The authors are greatly indebted to Professor Charles H. Blake, of the Biology Department, for calling our attention to this problem of melanins and for many profitable discussions on their occurrence and properties. One of us, W. R. S., is also indebted to the American Cancer Society for a predoctoral fellowship during this study.

EXPERIMENTAL

3-Benzyloxyacetanilide, XIV—*m*-Hydroxyacetanilide (9) (220 gm., 1.46 moles) was dissolved in 600 ml. of dry methanol containing 81.5 gm. (1.46

moles) of potassium hydroxide and methanol and water were removed under reduced pressure. Benzyl chloride (204 gm. or 1.6 moles), previously dried over calcium chloride, and 315 ml. of anhydrous ethanol were then added to the solid potassium phenoxide and the mixture was refluxed on a steam bath for 2 hours. The mixture, when cooled and filtered, yielded a brown solid, and the mother liquor yielded more after being diluted with 100 ml. of water. The combined precipitates were washed with 500 ml. of cold 1 N sodium hydroxide and then with ice water. The yield was 259 gm. or 74 per cent. A sample was alternately crystallized from benzene, ethyl acetate, and alcohol until a white product, which melted at 122.5–123.5° (corrected), was obtained. This material was very soluble in chloroform and acetone, was soluble in nitromethane, ethanol, and ethyl acetate, and was insoluble in petroleum ether, ether, cyclohexane, benzene, and carbon tetrachloride.

Analysis—$C_{15}H_{15}O_2N$. Calculated. C 74.68, H 6.27, N 5.81
Found. " 74.35, " 6.42, " 6.01

The above reaction was even better when the *m*-hydroxyacetanilide was refluxed with benzyl chloride in acetone that contained potassium carbonate. The yield was 85 per cent.

m-Benzyloxyaniline, XV—This compound was prepared by refluxing 145 gm. of the anilide, XIV, with 336 gm. (6 moles) of potassium hydroxide in 2 liters of 90 per cent alcohol for 6 hours. The alcohol was then removed by distillation and the residue was extracted with ether. The extract was washed, dried over Drierite, and distilled (171°, 2 mm.). When redistilled, this oil solidified on standing. The pure product melted at 61–62.5°. It was very soluble in ether, ethanol, acetone, chloroform, carbon tetrachloride, and 1 N hydrochloric acid, and was insoluble in benzene, cyclohexane, and petroleum ether.

Analysis—$C_{13}H_{13}ON$. Calculated. C 78.36, H 6.58, N 7.03
Found. " 78.24, " 6.53, " 6.84

The *p*-toluenesulfonyl derivative melted at 112–112.5° (corrected) and was soluble in acetone and chloroform, insoluble in ether, ethyl acetate, alcohol, benzene, cyclohexane, and alkali.

Analysis—$C_{20}H_{19}O_3NS$. Calculated. C 67.95, H 5.43, N 3.96
Found. " 67.95, " 5.33, " 4.08

Ethyl 3-Phenyl-6-benzyloxyindole-2-carboxylate, XVI—*m*-Benzyloxyaniline, 24.9 gm. or 0.125 mole, was diazotized in a solution of 8.7 gm. (0.126 mole) of sodium nitrite in 20 ml. of water, 150 gm. of ice, and 35 ml. of concentrated hydrochloric acid. This solution was then added at once to a solution of 27.5 gm. (0.125 mole) of ethyl *α*-benzylacetoacetate (10) in 190 ml. of alcohol, 40 ml. of water, and 15 gm. (0.375 mole) of sodium hydroxide. After 5 minutes, 300 ml. of water were added. The black oily precipitate was washed by decantation and then dried. This oil was dissolved in 50 ml. of dry alcohol and the solution saturated with dry hydrogen chloride. Refluxing half an hour caused the separation of ammonium chloride. Addition of water precipitated a dark resinous mass after a few hours. Extraction of this crude material with petroleum ether gave a light colored solid which could be crystallized from alcohol, ethyl acetate, carbon tetrachloride, or nitromethane. Eventually a 17 per cent yield of tiny white square plates that melted at 165–165.5° (corrected) was obtained.

Analysis—$C_{24}H_{21}O_3N$. Calculated. C 77.60, H 5.70, N 3.76
Found. " 77.37, " 5.86, " 3.59

3-Phenyl-6-hydroxyindole-2-carboxylic acid, VI—In a special creased flask (11) with inverted bottom, equipped with a mercury-sealed stirrer and reflux condenser, were placed 3.80 gm. (0.01 mole) of the ester, XVI, 4.1 gm. (0.03 mole) of anhydrous aluminum chloride, and 200 ml. of dry benzene. The mixture was refluxed with stirring for 1 hour. The contents were then poured on ice and acidified with hydrochloric acid. The ice-cold solution was immediately extracted with ether and the ether in turn with sodium hydroxide. Ice was added to the alkaline layer and the red alkaline solution titrated with concentrated hydrochloric acid until a copious white precipitate appeared. The precipitate was filtered, washed until neutral to litmus, and dried at reduced pressure. A light brown solid that melted at 170–175° was obtained. It was soluble in sodium carbonate but the neutralization equivalent was high, being 264 instead of the calculated 253. Furthermore, the amount of solid obtained was 2.62 gm., or 20 mg. more than the theoretical amount.

The above results indicated that some of the product had taken up oxygen. The 5 position was suspected and a test with antimony trifluoride for adjacent phenolic groups was made. The antimony trifluoride was dissolved in a saturated sodium chloride solution and the solution was neutralized with sodium carbonate solution until the precipitate which formed no longer dissolved. The solution was then filtered. The crude acid, VI, was dissolved in a volume of methanol equal to that of the inorganic solution. The two solutions were then warmed and mixed. A precipitate immediately formed, indicative of the diphenolic compound. The precipitate was filtered, washed with water to remove antimony and sodium salts, and then with methanol to remove organic acid. A small amount of black solid remained when the residue was dried.

That all of the compound had not been oxidized to a diphenolic product was indicated by the results of an attempt to esterify the crude acid, VI, (2.52 gm.) with dry hydrochloric acid in 200 ml. of alcohol. The temperature was maintained below 60° for 50 minutes. When the phenolic product was recovered by extraction and sublimation, the analysis suggested loss of nearly all of the carboxyl group. Accordingly the trace of ester was removed by saponification with 10 per cent sodium hydroxide, under nitrogen atmosphere, a few mg. of sodium bisulfite also being added in order to prevent oxidation. The neutral material, 6-hydroxy-3-phenylindole, recovered from these steps consisted of 200 mg. of a white solid that melted at 175–177° (corrected).

Analysis—$C_{14}H_{11}ON$. Calculated. C 80.33, H 5.30, N 6.69
Found. " 79.97, " 5.36, " 6.63

Two further attempts were made to obtain a pure ester from the crude acid, VI; one was with the silver salt-methyl iodide method and the other was with diazomethane. In general, neither of these processes was successful. The product was usually a brown solid. Sublimation at 2 μ and 150° yielded lighter colored material, some even white, but none that corresponded exactly with the value for the pure methyl ester. Each fraction was low in carbon and high in oxygen, indicative of contamination with the oxidized product.

4-Nitro-2-hydroxytoluene, XVIII—The synthesis of VII began with technical nitrotoluidine, XVII, and followed the method of Ullmann and Fitzenkam (12). The yellow crude product melted at 113–116°, and was used in the next step. The recorded value is 118°.

4-Nitro-2-benzyloxytoluene, XIX—The benzyl ether was made by refluxing overnight 8.5 gm. (0.056 mole) of XVIII with 5.8 gm. (0.042 mole) of anhydrous potassium carbonate and 7.6 gm. (0.06 mole) of dry benzyl chloride in 150 ml. of acetone. The mixture was stirred gently to prevent bumping. The product was washed with 1 per cent sodium hydroxide and then with water. The yield of crude ether was 74 per cent. Recrystallization successively from alcohol, petroleum ether, and methanol (decolorizing carbon used in the last) gave white microscopic needles which melted at 75.5–76° (corrected). The product was very soluble in ethyl acetate, benzene, and acetone, soluble in alcohol and methanol, and insoluble in water.

Analysis—$C_{14}H_{13}O_3N$. Calculated. C 69.12, H 5.39, N 5.76
Found. " 68.84, " 5.46, " 5.75

4-Amino-2-benzyloxytoluene, XX—On a steam bath 6.7 gm. (0.03 mole) of stannous chloride dihydrate, 8 ml. (0.1 mole) of concentrated hydrochloric acid, and 20 ml. of alcohol were heated to boiling in a beaker. Addition of 2.43 gm. (0.01 mole) of crude XIX caused a rapid exothermic reaction. In 10 minutes a copious white precipitate appeared. After 5 more minutes the beaker was placed on ice, and the pasty solid recovered by filtration. Addition of 10 per cent sodium hydroxide produced an oil which was extracted with ether. Addition of a small amount of concentrated hydrochloric acid then yielded 1.7 gm. (70 per cent) of a yellow solid. Sublimation at 1 mm. in a large test-tube gave first a yellow solid and then a pure white hygroscopic solid. The latter melted at 216.5–217° with decomposition. This hydrochloride of XX was soluble in hot alcohol and insoluble in alcohol, ether, benzene, acetone, and water.

Analysis—$C_{14}H_{16}ONCl$. Calculated. C 67.33, H 6.46, N 5.61
Found. " 67.21, " 6.38, " 5.84

Addition of alkali to the hydrochloride yielded an oil, XX, which distilled at 157° and 1 mm. and had a refractive index, $n_D^{25} = 1.6022$.

Analysis—$C_{14}H_{15}ON$. Calculated. C 78.84, H 7.09, N 6.57
Found. " 78.70, " 7.07, " 6.71

Ethyl 3-Phenyl-5-methyl-6-benzyloxyindole-3-carboxylate, XXI—This compound was made by the procedure used for XVI from 3 gm. (0.012 mole) of the crude hydrochloride of XX, 0.9 gm. of sodium nitrite, 24 ml. (0.024 mole) of 1 N hydrochloric acid, 1.45 gm. (0.036 mole) of sodium hydroxide in 5 ml. of water, and 2.65 gm. (0.012 mole) of ethyl benzylacetoacetate in 20 ml. of alcohol. The crude brown product amounted to 3.2 gm., 69 per cent yield. A cold methanol wash left a light brown solid which was crystallized from cyclohexane and then decolorized with carbon and recrystallized from the same solvent. The final yield was 1.8 gm. (39 per cent) of white microscopic needles that melted at 173.5–174.5° (corrected). It was very soluble in ethyl acetate, benzene, ether, and acetone, soluble in hot methanol, ethanol, or cyclohexane, and insoluble in petroleum ether.

Analysis—$C_{25}H_{23}O_3N$. Calculated. C 77.91, H 6.00, N 3.64
Found. " 77.97, " 6.13, " 3.74

3-Phenyl-5-methyl-6-hydroxyindole-2-carboxylic Acid, VII—This compound was made by the method used for VI from 1.0 gm. (0.0026 mole) of the ester, XXI, 1.05 gm. (0.0078 mole) of aluminum chloride, and 100 ml. of dry benzene. The yield was 585 mg. (84 per cent) of nearly white solid that melted at about 195°, and was very soluble in methanol, ethanol, ether, and acetone and insoluble in benzene and chloroform. Repeated crystallization from hot benzene and the use of decolorizing carbon would not remove all the color. The light tan needles melted at 205–205.5° (corrected) with decomposition. Analysis showed a small trace of ash still present.

Analysis—$C_{16}H_{13}O_3N$. Calculated. C 71.90, H 4.90, N 5.24
Found. " 71.34, " 4.92, " 5.19, ash 0.61

Oxidative Polymerization of VII—0.5 gm. (0.00188 mole) of VII was dissolved in 40 ml. of glacial acetic acid. A small trace of insoluble residue was removed by filtration. A solution (5 ml.) of vanadium pentoxide (1 to 2 mg.) in 1 N hydrochloric acid and 2 ml. (0.001 mole) of 0.5 N potassium chlorate was added. The solution immediately became jet-black. The next day the reaction mixture was filtered and the precipitate washed thoroughly with water. When dried, the purple-black solid, Fraction *a*, weighed 0.1 gm.

Analysis—Found, C 74.87, H 4.42, N 5.44, ash, trace

The filtrate was allowed to stand 5 more days and more polymer was collected. The dark brown solid, Fraction *b*, was washed and dried as before. This fraction had a neutralization equivalent of the order of 10,000. When allowed to stand overnight in 10 per cent sodium hydroxide, only a little of Fraction *b* dissolved. None of Fraction *a* dissolved. Subsequently, after being filtered and washed, part of Fraction *b* dissolved to give a dark green solution. The residue was red. Carbon dioxide reprecipitated the polymer. Fraction *b*, C 73.11, H 4.95.

Ferric chloride (4.0 ml. of 1 N solution) was used as the oxidizing agent in place of the chlorate. The black solid isolated by the end of 1 day was 0.137 gm. C 76.39, H 5.04, N 5.43, ash, trace.

In alcohol solution the quantities of reagents were the same as in acetic acid. Potassium chlorate yielded 0.054 gm. of a red solid after standing overnight. C 74.77, H 4.59, N 5.06, ash, trace. Ferric chloride, by the end of 1 day had produced 0.073 gm. of a black solid. C 74.09, H 5.28, N 4.98.

When oxidizing agents were not used, no precipitation occurred.

N-Methyl-m-benzyloxyaniline—Sodium sand (4.6 gm. or 0.2 mole) was made by vigorous agitation in hot xylene in the high speed stirring apparatus (11) under an atmosphere of nitrogen. The xylene was cooled to 60° and pure dry *m*-benzyloxyacetanilide, XIV, (48.2 gm. or 0.2 mole) was added with rapid stirring to insure thorough mixing. The temperature was raised to 100° and held at 100–110° for 1 hour. A rapid evolution of hydrogen occurred and the solution gradually became homogeneous. The heater was then removed and 18.9 gm. (0.15 mole) of dry methyl sulfate were added in small portions. When this highly exothermic reaction was completed, the solution was stirred an additional 15 minutes and then cooled and filtered. Xylene was removed from the filtrate by distillation at reduced pressure to give an oil. Meanwhile the jelly-like product on the filter was dissolved in water and more of the oil recovered. To the combined oil fraction 55 gm. of potassium hydroxide in 500 ml. of 90 per cent alcohol were added. The mixture was refluxed for 48 hours.

The alcohol was removed by distillation and the residue extracted with ether. The ether extract was washed, dried with sodium sulfate, and distilled. At 156–165° and 1 mm., 40 gm. or 94 per cent of a straw-colored liquid were obtained. This product was dissolved in acetone, cooled to −72°, and filtered through a bed of solid carbon dioxide. The filtrate was then distilled twice and the N-methyl-*m*-benzyloxyaniline collected at 144–145°, 1 mm., as a pale yellow liquid; $n_D^{25} = 1.5960$.

Analysis—$C_{14}H_{15}ON$. Calculated. C 78.84, H 7.09, N 6.57
Found. " 78.90, " 7.19, " 6.82

The acetyl derivative melted at 70–72°. It was soluble in all common solvents except cyclohexane and petroleum ether.

Analysis—$C_{16}H_{17}O_2N$. Calculated. C 75.27, H 6.71, N 5.49
Found. " 75.26, " 6.80, " 5.59

The picrate was obtained when a boiling dilute hydrochloric acid solution was mixed with a boiling saturated aqueous solution of picric acid. The product was crystallized from alcohol and then from ethyl acetate to give large yellow-brown, four-sided prisms that melted at 138–138.5° (corrected).

Analysis—$C_{20}H_{18}O_8N_4$. Calculated. C 54.30, H 4.10, N 12.67
Found. " 54.40, " 4.19, " 12.80

N-Nitroso-N-methyl-m-benzyloxyaniline—The above compound (1.0 gm. or 0.0047 mole) was partially dissolved in 82 ml. of 0.1 N hydrochloric acid. The mixture was cooled to 15° and an aqueous solution of 0.4 gm. (0.006 mole) of sodium nitrite was added rapidly with stirring. After 10 minutes the orange-colored solution was extracted with ether. From the extract 0.9 gm. (88 per cent) of a brown-red solid was recovered. Crystallization from 1:1 ether-petroleum ether and then from methanol yielded orange-colored crystals that melted at 62–62.5°. It was soluble in chloroform, ether, benzene, ethyl acetate, and hot cyclohexane, slightly soluble in methanol and alcohol, and insoluble in cyclohexane and petroleum ether.

Analysis—$C_{14}H_{14}O_2N_2$. Calculated. C 69.40, H 5.82, N 11.57
Found. " 69.70, " 6.01, " 11.58

Reduction of this compound with zinc dust and acetic acid failed to give the hydrazine.

Ethyl 3-Phenyl-6-methoxyindole-2-carboxylate—*m*-Anisidine, 6.1 gm. or 0.05 mole, was subjected to a Japp-Klingemann condensation with 11 gm. (0.05 mole) of ethyl benzylacetoacetate, sodium nitrite, hydrochloric acid, and sodium hydroxide in the same manner and the same proportions as were used in the preparation of XVI. The resinous product was crystallized alternately from alcohol and ethyl acetate until white needles, m.p. 176–176.5°, were obtained.

Analysis—$C_{18}H_{17}O_3N$. Calculated. C 73.22, H 5.79, N 4.74
Found. " 73.24, " 6.08, " 4.64

When the ether was refluxed with aluminum chloride in chlorobenzene, a black solid was obtained.

SUMMARY

6-Hydroxy-3-phenylindole-2-carboxylic acid, similar in structure to a possible intermediate in polymerization of a hydroxyphenylalanine to melanin except for the stabilizing phenyl group, has been prepared and found to undergo easy oxidation at the 5 position to give probably the 5,6-dihydroxy compound.

The 5 position has been blocked by a methyl group and the corresponding 5-methyl-6-hydroxy-3-phenylindole-2-carboxylic acid can be isolated. It undergoes polymerization in the presence of mild oxidizing agents to give a melanin-like product.

The results are interpreted as showing that the 5 position is not necessarily involved in the polymerization of a 5,6-dihydroxyindole to melanin, since a quinomethane structure that involves the 6 position would be sufficient to give the polymer.

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FACTORS INFLUENCING THE AGGLUTINATION OF RED BLOOD CELLS, RED BLOOD CELL STROMA, AND LYMPHOCYTES*

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In a recent review of the literature (1, 2), many of the problems dealing with cellular agglutination caused by ricin are presented and discussed. Although this phenomenon was first described in 1888 by Stillmark, reactions involved in the agglutination of cells are poorly understood. The present report is concerned with factors which affect agglutination. Data are presented which show that erythrocyte, erythrocyte stroma, and lymphocyte agglutination may be influenced by a large number of different materials and conditions.

Methods

The degree of agglutination of red blood cells was determined by a method described by Cannan.[1] This procedure is based on the ability of unagglutinated red cells to pass through filter paper. Human red cells (type O) were obtained from the hospital blood bank and were washed with buffered saline[2] three times with the aid of the centrifuge. A 5 per cent suspension of these cells was prepared in buffered saline and the mixture was filtered through Whatman No. 2 paper to remove clumped red cells.

An agglutinin-rich precipitate was obtained by dialyzing an aqueous extract of cold-pressed castor bean pomace against running tap water maintained at 2°. This precipitate was suspended in saline and the insoluble material was removed by centrifuging. The agglutinin-rich supernatant served as a stock solution and retained its activity while frozen. A dilute solution was prepared from the stock solution at weekly intervals and was kept at 5°.

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* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Virginia. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

[1] Cannan, R. K., personal communication.

[2] The buffered saline solution (pH 6.9) was prepared as follows: a stock solution containing 35.0 gm. of K_2HPO_4 and 13.5 gm. of KH_2PO_4 was dissolved in a liter of water; 8.2 gm. of NaCl and 66 ml. of the stock buffer solution were mixed and brought to a liter with distilled water. The phosphate concentration of this solution was 0.02 M.

The degree of hemagglutination was determined by the following procedure: 1 ml. of washed cells and 3.5 ml. of the buffered saline were mixed in a 25 ml. Erlenmeyer flask; 0.5 ml. of the agglutinin-containing solution was added with continuous shaking. Shaking is essential for reproducible results. A control tube was prepared in which saline was substituted for the agglutinin solution. The mixture was transferred to a 10 × 100 mm. test-tube, which was plugged with a cellophane-covered rubber stopper. The tubes were placed in specially constructed racks which were rotated 4 times a minute at room temperature for 30 minutes, when the tubes were immersed in ice water for 10 minutes and the contents filtered through Whatman No. 2 paper in the cold room. This procedure was found necessary for constant results, since large variations in the number of filtered cells were encountered if filtration was done at room temperatures. The filtrate containing the unagglutinated red cells was brought to room temperature and 1 ml. was added to 5 ml. of a saponin solution (0.05 per cent). The color intensity of the hemolyzed solutions was read in a Klett-Summerson colorimeter with Filter 54. All determinations were done in duplicate. A straight line relationship is obtained between the degree of hemagglutination with varying amounts of agglutinin.

In order to test the effect of proteins[3] and other materials on hemagglutination, a standard procedure was adopted in which these materials were first dissolved in buffered saline and varying quantities were incubated with the diluted red blood cell suspension for 10 minutes. Inconsistent results were obtained with shorter incubation periods. The buffered saline prevented any appreciable change in pH which might be caused by the addition of protein and other material.

Human red blood cell stroma was prepared by a procedure described by Bernstein *et al.* (5). The dried stroma was first ground in a mortar, then suspended in physiological saline and treated for 2 minutes in a Waring blendor, run at top speed in the cold. The suspension was centrifuged at very low speeds for a few minutes and stroma particles of uniform size were present in the supernatant. These suspensions were prepared daily. In order to determine the degree of stroma agglutination, the routine procedure finally adopted was as follows: 3 ml. of stroma suspension, 1 ml. of agglutinin-containing solution, and 1 ml. of a solution containing the test material or saline were rotated for 15 to 30 minutes and centrifuged at high speed. The supernatant was discarded. To the packed stroma, 2 drops of a saline-eosin solution were added and the mixture was well stirred. The suspension was diluted 20 times with saline-eosin in a white cell pipette and the agglutinated particles were counted in a counting chamber. Occasionally large clumps of agglutinated stroma were formed, which were responsible for unreliable results.

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[3] The human plasma fractions obtained from the Department of Physical Chemistry, Harvard University, were prepared by ethanol precipitation. The rat and goat fractions were prepared in this laboratory (3, 4). Crystallized bovine albumin was obtained from the Armour Laboratories and the concentrated commercial albumin manufactured by the Cutter Laboratories was purchased on the open market.

To obtain lymphocytes, the thymus of a 60 day-old rat was teased while suspended in cold saline. The saline suspension of the tissue was filtered through muslin and the white cells were obtained in the filtrate. The cells were diluted so that the mixture contained from 500 to 1000 white cells per c.mm. The lymphocyte suspension (4 ml.) was incubated with agglutinin (1 ml.) and the test material for 30 minutes. The non-agglutinated cells were counted and the percentage agglutination was calculated from a control lymphocyte-saline suspension.

Results

Effect of Plasma and Plasma Fractions on Agglutination of Human Red Blood Cells by Castor Bean Agglutinin—Representative data are plotted in Fig. 1, *A* for human plasma and several of its fractions which inhibit hemagglutination. A potent antiagglutinin factor is present in Fractions IV-1, IV-4, and II+III; small amounts of these protein fractions are capable of preventing hemagglutination almost completely. Whole plasma and a commercial 25 per cent albumin solution (Cutter) are also effective antiagglutinins.

The Cutter albumin solution contains approximately 95 per cent albumin. According to electrophoretic analysis, the proteins of the Cutter product used consist of 95 per cent albumin and small amounts of various globulins. In experiments discussed below, it will be seen that crystalline human plasma albumin has little effect or increases the extent of the agglutination. The acetyltryptophan, which is used as a stabilizing agent in the Cutter albumin, has no effect on hemagglutination. It, therefore, follows that the agglutinin-inhibitor is associated with one or more of the globulins which are present as impurities.

Rat plasma and several of its fractions act as antiagglutinins for human red cells (Fig. 1, *B*). Comparatively large amounts of rat plasma proteins are necessary to inhibit hemagglutination. Fraction IV-4 is the most potent antiagglutinin.

Crystalline human and bovine albumin and electrophoretically pure goat albumin do not influence hemagglutination. In the presence of relatively large amounts of the albumin (6 to 20 mg.), there is a gradual enhancement in the percentage agglutination. These findings are characteristic of mixtures in which the control percentage agglutination is relatively low. When the control percentage hemagglutination is at 60 per cent or over, addition of albumin causes a slight decrease in the percentage agglutination.

Effect of Casein, Acacia, Sodium Nucleate, and Gelatin on Human Red Blood Cell-Agglutination Mixture (Fig. 2)—Casein (Labco) and acacia exert an inhibitory effect on hemagglutination. The results for casein are similar to those obtained for Fraction II (Fig. 1, *A*). The magnitude of these changes depends on the initial degree of hemagglutination. Relatively small amounts of the nucleate are markedly effective in increasing the percentage agglutination. Gelatin is the most effective protein, in terms of nitrogen content, in enhancing hemagglutination.

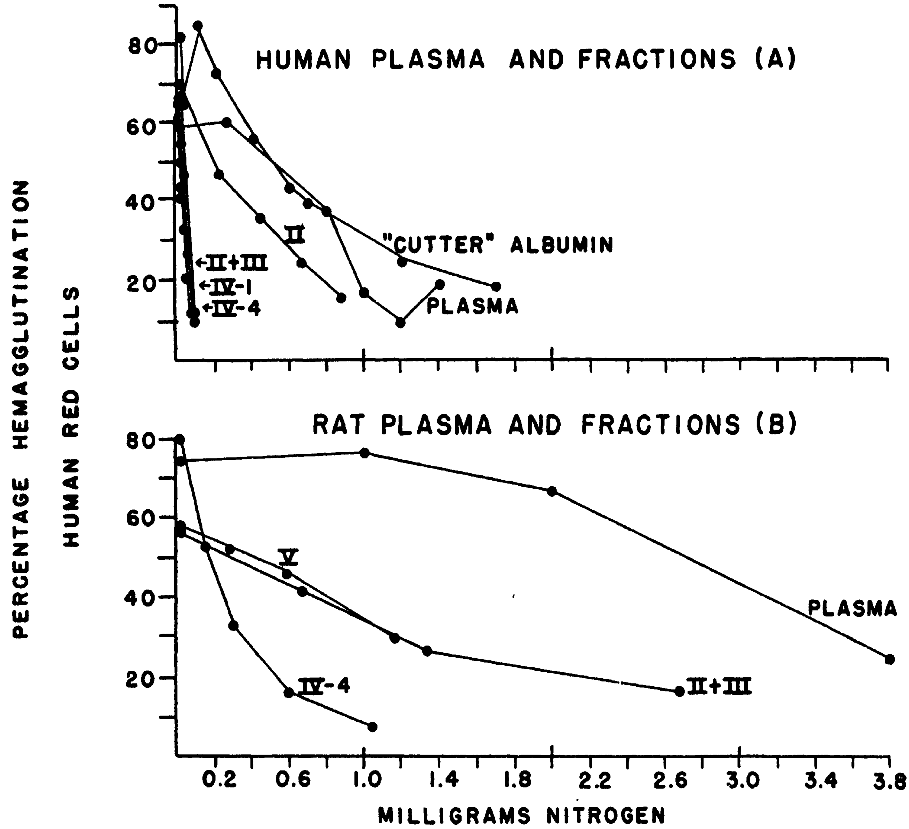

FIG. 1. Effect of human (*A*) and rat (*B*) plasma and plasma protein fractions on hemagglutination.

Control experiments were conducted to determine whether the various test substances alone caused clumping of red cells. Under the conditions of these experiments, the red cells were not affected.

Effect of Albumin on Agglutination and Hemolysis of Rat Red Blood Cells

(*Table I*)—Red blood cells of the rat are unsatisfactory for hemagglutination studies because of their marked fragility in saline. It was found that hemolysis could be minimized by goat, bovine, human, and Cutter albumins. The percentage agglutination in the presence of 0.1 per cent of goat, bovine, and human albumins does not differ greatly. The globulins of the Cutter product are responsible for marked inhibition.

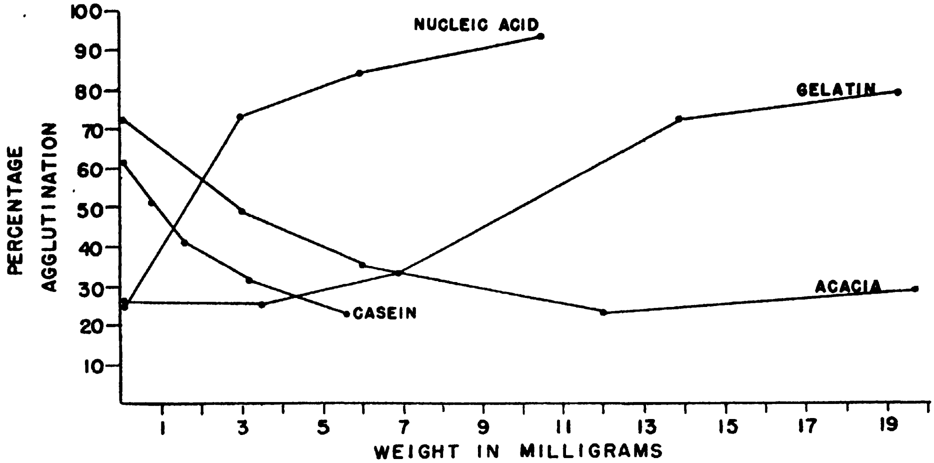

FIG. 2. Effect of casein, acacia, nucleate, and gelatin on hemagglutination

TABLE I

Effect of Various Types of Albumin on Agglutination and Hemolysis of Red Blood Cells of Rat

3.5 ml. of buffered saline containing 3.5 mg. of albumin were mixed with 0.5 ml. of agglutinin solution; 1 ml. of diluted rat red cells was added and the mixture treated according to the regular procedure. The red cells were washed and diluted with buffered saline containing 0.1 per cent of the respective albumin. These experiments were done on samples of the same rat red cells and run at the same time.

Albumin	Agglutination	Hemolysis*
	per cent	*per cent*
Goat†	82	3
Bovine†	78	5
Human†	69	6
Cutter	22	10
None		33

* The degree of hemolysis was determined in the supernatant of the centrifuged red cell filtrates.

† Electrophoretically pure or crystalline.

Effect of Sequence of Mixing Components—In the method adopted for determining the degree of hemagglutination, human red cells are added to solutions of the test substances and the agglutinin solution is added. When the sequence is varied by adding the red cells last, both Fraction IV-4 and casein are responsible for a greater inhibition of hemagglutination. The order of mixing has no effect in those experiments in which crystalline human albumin, nucleate, gelatin, or acacia is added.

Red Blood Cell Stroma-Agglutinin Reaction—Agglutinin undoubtedly reacts with or is adsorbed on one or more constituents of the red cell to cause agglutination. To study one phase of this problem, a mixture of stroma and agglutinin is incubated, the stroma is removed by centrifugation, and the amount of the agglutinin remaining in the supernatant is determined by the degree of hemagglutination. The results of three experiments in which the amounts of agglutinin and stroma were varied are shown in Fig. 3. The three curves differ in shape, owing to the large variation in the amount of agglutinin present. It is clear that stroma removes appreciable amounts of agglutinin. Traces of agglutinin appear to be present even after incubation with large amounts of stroma. These data do not offer an explanation for the mechanism of agglutination but they do indicate that the red blood cell stroma reacts with or is affected by agglutinin.

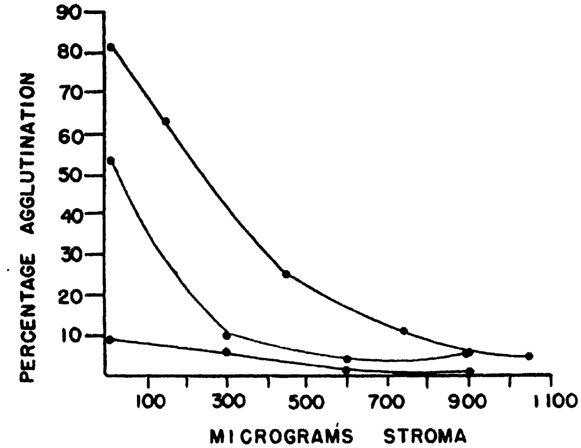

FIG. 3. Effect of incubating red blood cell stroma with varying amounts of agglutinin as determined by the degree of hemagglutination in the stroma-free supernatant.

The above evidence indicates that stroma and agglutinin probably form a complex. The dissociation of the stroma-agglutinin complex was studied by extracting it at different hydrogen ion concentrations and determining the agglutinin content of the extracts. The agglutinin appears to be firmly bound by stroma between pH 5.2 and pH 7.7, since none is extracted at these hydrogen ion concentrations. Relatively high acidic and basic conditions are necessary to dissociate the stroma-agglutinin complex.

Agglutination of Stroma—During the course of the experimental work with stroma, it was observed that this material was readily agglutinated by the castor bean agglutinin. In order to study this phenomenon quantitatively, varying amounts of agglutinin were added to constant amounts of a special preparation of stroma of uniform size and the mixture was rotated; the clumps of agglutinated stroma were counted. A progressive increase in the percentage agglutination is obtained with increasing amounts of agglutinin.

Fraction IV-4 does not completely inhibit stroma agglutination despite the presence of relatively large amounts of this fraction (10 mg.). It appears that factors other than those present in stroma are involved in the agglutination of red cells, since the latter are extremely sensitive in the presence of this fraction. The effect of crystalline human and bovine plasma albumin on the agglutination of stroma is similar to that observed with red blood cells.

TABLE II

Agglutination of Lymphocytes in Presence of Crystalline Human Plasma Albumin, "Cutter" Albumin, and Human Fraction IV-1

Agglutinin	Per cent agglutination			Agglutinin	Per cent agglutination	
	Control	Albumin, 10 mg.			Control	Fraction IV-1, 0.3 mg. N
		Crystalline	Cutter			
γN				γN		
0.20	81	73	19	0.03	25	8
0.25	82	77	31	0.11	34	13
0.33	87	84	41	0.14	43	13
0.40	92	86	44	0.20	63	13
0.45	92	88	46	0.33	79	25

The influence of agglutinin on agglutination of stroma may be determined quantitatively between pH 6.9 and 7.7. Marked clumping is seen at lower hydrogen ion concentrations, while more alkaline reactions cause inhibition.

Agglutination of Lymphocytes (Table II)—Lymphocytes of rat thymus are very sensitive to the castor bean agglutinin. According to the control data, extremely small amounts of agglutinin have a marked agglutinating effect on lymphocytes of rat thymus. The crystalline albumin has practically no effect on agglutination, while the commercial Cutter product has an inhibitory effect. Fraction IV-1 is an effective inhibitor of lymphocyte agglutination. These results show that inhibitors of red blood cell agglutination are also effective for lymphocytes.

SUMMARY

Procedures are presented for quantitatively determining the agglutination of red blood cells, red blood cell stroma, and lymphocytes by castor bean agglutinin.

The agglutination of human red cells is inhibited by (*a*) human plasma and plasma protein fractions, particularly Fractions IV-1 and IV-4; (*b*) commercial concentrated albumin solutions; (*c*) rat plasma and several of its protein fractions; and (*d*) casein and acacia. Hemagglutination is not affected or is slightly increased by crystalline human and bovine albumin and electrophoretically pure goat and rat albumin.

Sodium nucleate and gelatin increase the degree of hemagglutination.

Agglutination of rat red cells is inhibited by rat plasma and its globulin-rich fractions. Hemolysis is prevented by small amounts of pure plasma albumins.

The sequence of mixing agglutinin, red cells, and test materials may influence the degree of hemagglutination.

Red cell stroma removes agglutinin from solution and is readily agglutinated at the same time. The stroma-agglutinin complex appears to be stable between pH 5.2 and 7.7. This agglutination is inhibited by Fraction IV-4 and not influenced by albumin.

Lymphocytes of rat thymus are readily agglutinated. Fraction IV-1 and commercial plasma albumin inhibit agglutination.

The evidence presented in this paper does not permit any postulation of the mechanism of agglutination.

The authors are indebted to Caroline Ball and Barbara S. Willets for technical assistance.

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CODECARBOXYLASE NOT PYRIDOXAL-3-PHOSPHATE

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That pyridoxal, in a phosphorylated form, is the coenzyme of amino acid decarboxylases (1–4) has been well established. Other enzymes, including transaminase (5, 6), have also been found to be activated by this compound. The coenzyme has been prepared by chemical phosphorylation of pyridoxal and purified as the barium salt, whose properties have been reported (7). Because the method of synthesis did not reveal the location of the phosphate, its position on the molecule was not specified.

Recently reports have been published which state that synthetic pyridoxal-3-phosphate, in the form of the crystalline acetal, is the coenzyme of the amino acid decarboxylases (8–10), although it lacks activity for the transaminases (11). We have previously (12) called attention to data which demonstrate that the barium salt of pyridoxal phosphate which we had prepared and purified (7) exhibited much greater coenzyme activity per unit weight than did a sample of pyridoxal-3-phosphate acetal prepared and characterized by Heyl, Harris, and Folkers (13).[1] However, it has again been claimed (14) that the pyridoxal-3-phosphate acetal is the coenzyme of amino acid decarboxylases.

It appeared that the best way to settle the difference of opinion was to test the compounds in question with a single enzyme preparation under identical conditions and thus let the enzyme decide which compound possessed the greater activity. For this purpose Professor Karrer kindly furnished us a sample of the crystalline pyridoxal-3-phosphate acetal prepared by Karrer and Viscontini (8), to be tested along with the pyridoxal-3-phosphate acetal prepared by Heyl, Harris, and Folkers (13) and the pyridoxal phosphate barium salt of unspecified structure which we had prepared (7).

The data in Table I show that for tyrosine decarboxylase enzyme 1 mg. of barium salt of pyridoxal phosphate prepared by us is equivalent in activity to 1130 mg. of pyridoxal-3-phosphate acetal prepared by Karrer and Viscontini and to 1740 mg. of the pyridoxal-3-phosphate acetal prepared by Heyl, Harris, and Folkers (with use of values approaching Q_{CO_2}

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[1] Kindly supplied by the Research Laboratories of Merck and Company, Inc.

= 120, which is the most accurate portion of the assay curve). Since our preparation of the barium salt contains only 32 per cent bound pyridoxal (7), whereas the 3-phosphate acetal contains 59 per cent (11), on a molar basis our barium salt is 2000 and 3000 times more active than pyridoxal-3-phosphate. The slight activity observed in the pyridoxal-3-phosphate preparations may result from a small amount of transphosphorylation, either chemical or enzymatic, or possibly represent a small degree of replacement of function. The latter seems unlikely in view of the lack of activity of 3-phosphate with the enzyme preparation used previously (12). In any event, it seems evident that pyridoxal-3-phosphate is not the coenzyme of tyrosine decarboxylase.

TABLE I

Activity of Pyridoxal Phosphate Preparations in Tyrosine Decarboxylation

Sample	Amount added per 3 ml.	Q_{CO_2} tyrosine
	γ	
Barium salt of pyridoxal phosphate (Sample 50-4, Gunsalus *et al.* (7))	None	24
	0.0564	300
	0.0376	264
	0.0188	208
	0.0136	126
	0.0094	90
	0.0037	42
Crystalline pyridoxal-3-phosphate acetal prepared by Karrer and Viscontini (8)	150	276
	15	120
	1.5000	60
	0.1500	24
	0.0150	24
	0.0015	24
Pyridoxal-3-phosphate acetal prepared by Heyl, Harris, and Folkers (13)	230	222
	23	120
	2.3000	48
	0.2300	18

The rate of tyrosine decarboxylation was determined with 1 mg. per cup of a vacuum-dried preparation of *Streptococcus faecalis* R grown in the absence of members of the vitamin B_6 group (15) essentially as previously described (2) except that the measurements were made at 37° and the interval from 5 to 20 minutes after the addition of tyrosine was used to calculate the Q_{CO_2}.

SUMMARY

A comparison of the activity in stimulating tyrosine decarboxylase of pyridoxal-3-phosphate prepared by Karrer and Viscontini, the pyridoxal-3-phosphate prepared by Heyl, Harris, and Folkers, and the pyridoxal

phosphate of unspecified structure which we had prepared shows that only the latter has marked activity. Therefore it is evident that pyridoxal-3-phosphate is not the coenzyme of tyrosine decarboxylase.

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THE METABOLISM OF PARENTERALLY ADMINISTERED AMINO ACIDS

I. GLYCINE*

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A considerable body of information has been accumulated concerning the metabolism of amino acids *in vitro*. However, virtually no information is available concerning the capacity of the mammalian organism to metabolize individual amino acids or mixtures thereof, although such data should be of interest both for comprehension of the pathways of amino acid metabolism and for the formulation of amino acids mixtures for clinical use. The experiments reported herein are the first of a series of such studies now in progress.

Woodyatt and his colleagues (2–5), and numerous investigators since, studied the metabolism of glucose by measuring the rate of intravenous infusion at which it became possible to attain a steady state, *viz.* a constant blood glucose concentration. This approach is not feasible for amino acids, since virtually no renal threshold exists and, in addition, the rate of entry of amino acids into cells is variable and, in the case of skeletal muscle cells, quite slow. Therefore, in the present studies, whenever a single amino acid is considered, the rate of urea formation is regarded as equivalent to the rate of amino acid metabolism. The technique of constant rate intravenous infusion at rates sufficient to saturate deaminating mechanisms has been used. Since the primary purpose of the present work was to establish conditions whereby rates of metabolism may be studied, the methods are described in detail. Accessory findings, not directly related to the study of rates of metabolism, are also reported, since these are necessary for the evaluation of the other metabolic data.

Glycine was the first amino acid selected for study. The data obtained are to serve as reference points in studies of the metabolism of other amino acids and combinations thereof which are now in progress.

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* A preliminary report of this work has been published (1).

† The data presented herein were taken, in part, from a thesis submitted by Henry Kamin to the Graduate School of Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

EXPERIMENTAL

Female mongrel dogs, weighing from 5 to 15 kilos each, were used throughout the study. They were maintained on a mixed stock diet which they were allowed to eat *ad libitum*. All dogs were anesthetized with dial (diallylbarbituric acid, Ciba) or nembutal (sodium pentobarbital, Abbott), the level of anesthesia being maintained on as light a plane as was commensurate with the prevention of undue activity. When it was necessary to sacrifice control dogs for the purpose of obtaining tissue specimens, rapid ether administration was used.

Blood samples were obtained by venipuncture, usually of the jugular vein, except for postmortem blood, which was obtained by heart puncture. Urine was obtained by means of an indwelling catheter, the bladder being rinsed thoroughly at each collection.

The glycine solutions, which were delivered into the radial vein, varied in concentration from 2 to 9 per cent. Glucose solutions ranged in concentration from 5 to 15 per cent, and sodium chloride and sodium sulfate, when infused to induce diuresis, were administered as 0.45 and 1.5 per cent solutions, respectively. An amount of sodium chloride such as to make the final concentration 0.3 per cent was added to all solutions to replace possible salt loss accompanying diuresis. Solutions were usually infused by gravity from a previously calibrated 700 ml. graduated Pyrex infusion bottle equipped with a Murphy drip; the rate of flow was controlled by a tunnel clamp. In experiments in which constancy of rate of infusion was critical, an electrically driven constant rate intravenous pump was used.

Analytical Methods—All blood analyses, unless otherwise stated, were performed upon plasma, which was separated and deproteinized immediately after collection. Urine specimens were acidified to pH 2 to 3 with 18 N sulfuric acid and preserved with toluene.

Tissue filtrates for α-amino N determinations and microbiological determination of specific non-protein amino acids were prepared by grinding the tissue in a Waring blendor with the appropriate protein precipitant, heating to 70–80° on a steam bath, and filtering while hot. 1 per cent picric acid was used for the preparation of filtrates for α-amino N determination; freshly prepared 1 per cent tungstic acid was used for microbiological amino acid analysis. Rat livers were deproteinized immediately upon removal, while tissue specimens from dogs were frozen rapidly in dry ice and acetone and stored at −5° to −8° until analyzed.

Since in most cases glycine constituted by far the greatest fraction of the total α-amino N determined, it was necessary to perform blood and urine analyses under conditions which would insure maximum recovery of that amino acid. The procedure of Hamilton and Van Slyke (6) for the determination of α-amino N in plasma was found to be directly applicable, with glycine recoveries of 95 to 97 per cent of the theoretical being obtained. The urine procedure of Van Slyke *et al.* (7), however, gave only 70 to 80 per cent of the theoretical yield from glycine. This procedure was therefore modified by heating in the presence of ninhydrin for 20 minutes at pH 4.7 instead of 10 minutes at pH 2.5. Glycine recovery under the modified conditions was 93 to 95 per cent of theoretical; however, normal control urines analyzed under these conditions gave considerably higher results, often as much as 100 per cent higher than under the conditions of Van Slyke *et al.* (7).

For determination of plasma urea N, a spectrophotometric modification of the method of Karr (8) was employed. Since excess tungstate precipitated and inactivated urease, protein-free plasma filtrates were prepared with one-half the quantities of tungstate and acid recommended for whole blood. Urine urea N was determined by incubation of aliquots of permutit-treated urine with buffer and urease, followed by direct nesslerization. Results by this method showed consistently good agreement with the aeration-titration method of Van Slyke and Cullen (9). All colorimetric determinations were performed with a Coleman junior spectrophotometer.

Plasma non-protein nitrogen determinations were performed by a spectrophotometric modification of the Koch-McMeekin method (10), and urine total nitrogen determinations were performed by a macro-Kjeldahl procedure.

Microbiological amino acid determinations, except for glycine and glutamic acid, were performed by the method of Stokes *et al.* (11), with sodium citrate employed as the chief buffer salt in place of sodium acetate. L-Leucine was employed instead of synthetic DL-leucine, since it was found, as previously noted by Hegsted and Wardwell (12), that synthetic leucine contained significant quantities of isoleucine. Glycine was determined microbiologically by the method of Shankman *et al.* (13), and glutamic acid by the method of Dunn *et al.* (14). All samples were adjusted to pH 6.8 before addition to the basal medium, and final assay was performed by electrometric titration to pH 6.8 after a suitable incubation period. Urine samples were analyzed microbiologically with no treatment other than dilution and adjustment of the pH; protein-free filtrates of plasma for microbiological assay were prepared by the method of Hier and Bergeim (15).

Determination of extracellular fluid volume was made by intravenous injection of 20 mg. per kilo of body weight of sodium thiocyanate and measurement of plasma thiocyanate concentration after an equilibration period of at least 1 hour. Thiocyanate determinations were performed by a spectrophotometric modification of the method of Barker (16). The relative concentration of thiocyanate in tissues and plasma was used as a measure of extracellular fluid volume in tissues.

Total body water was considered to be 70 per cent of body weight; deviations from the initial body water content during the course of the experiment were calculated by adding the difference between fluid intake and urine output to the initial calculated water volume. The total body urea N content was calculated by multiplying the plasma concentration in mg. per liter by the liters of body water. The total α-amino N in extracellular fluid was calculated by similarly multiplying plasma α-amino N concentration by the extracellular fluid volume.

Plasma and urine inorganic P was determined by the method of Fiske and Subbarow (17), chloride by the method of Van Slyke and Hiller (18), and plasma carbon dioxide-combining capacity by the method of Van Slyke and Cullen (19).

Results

Toxicity of Glycine—Glycine infused at rates higher than 1 mg. of N per kilo per minute was invariably found to be lethal, but after varying periods of time. The time was inversely proportional to the rate of administration, so that toxicity depended upon the total dosage, rather than upon the duration of the infusion. At or below this apparently critical rate, the toxicity of glycine was variable; of twelve animals infused with glycine, or glycine plus glucose, at a rate of about 1 mg. of N per kilo per minute, eight expired after receiving doses of 360 to 1301 mg. of N per kilo (average, 774), and four survived doses ranging from 703 to 1970 mg. of N per kilo (average, 1212). The relationship between rate of glycine administration and time, dosage, and other factors with which correlation was attempted is illustrated in Table I.

As can be seen from Table I, glycine toxicity can be correlated better with total dosage than with any other quantity listed. It should be noted at this point that the fluid intake accompanying glycine infusion bore no quantitative relationship to toxicity; there were no deaths among a group of controls infused with comparable quantities of glucose, sodium chloride, and sodium sulfate solutions. Since, as will be seen further, the maximum rate of urea formation from glycine is of the order of 0.8 mg. of N per kilo per minute, it is possible that the variations in glycine toxicity in the neighborhood of that rate of administration may be related to that finding.

In all cases, although the time interval between the appearance of characteristic symptoms and the death of the animal varied, the pattern of symptoms was quite consistent and could readily be recognized by observers. The first sign of toxicity was usually nausea and vomiting. It is not believed that this symptom is entirely specific, since it was occasionally observed in control animals. Nausea and vomiting had been observed by other workers (20–23), but in their experiments, this symptom disappeared and recovery ensued upon the discontinuation of the infusion. It must be presumed, therefore, that this symptom occurs during a reversible stage of glycine intoxication and is a forerunner rather than an integral part of the irreversible syndrome to be described.

At varying intervals after the appearance of nausea and vomiting, respiration became slower and more abdominal in character. As time progressed this change became more marked until, near the end, respiration consisted of occasional convulsive gasps, with marked contraction of the diaphragm and abdominal muscles, alternating with prolonged periods of apnea. Cyanosis, hypothermia, and oliguria or anuria marked this terminal stage. Once respiratory changes had been noted, discontinuation of the infusion and the administration of coramine, metrazol, or adrenalin failed to produce recovery. The interval between the appearance of respiratory distress and the death of the animal varied between several

TABLE I

Quantitative Aspects of Glycine Toxicity

The results are averages of the determinations performed. The figures in parentheses refer to the range.

	Varying rates (3 dogs)	Medium rate (7 dogs)	High rate (2 dogs)	Low rate* (4 dogs)
Rate of administration, *mg. N per kg. per min.*	1.4–5.1	2.9 (2.7–3.1)	7.75 (7.7–7.8)	0.95 (0.84–1.1)
Time until death or terminal symptoms, *min.*	225 (216–236)	242 (138–236)	91 (83–99)	1052 (775–1301)
Glycine N infused, *mg. per kg.*	592 (476–691)	685 (400–910)	689 (605–772)	1026 (697–1310)
Glycine N accumulated in cells, *mg. per kg.*	126 (122–128)	92† (70–121)	105 (94–116)	23 (0–89)
Plasma α-amino N, terminal, *mg. %*	73 (59–83)	89‡ (51–135)	134 (124–144)	22 (12–33)
Urea N formed, *mg. per kg.*	188 (157–229)	233‡ (142–395)	67 (49–84)	949 (519–1270)
Liver α-amino N, *mg. per 100 gm.*		127 (88–145)	147§	58§

* Includes only those dogs for which complete data were available.

† Four dogs.

‡ Five dogs.

§ One dog.

minutes and 2 or 3 hours. However, several animals expired quite suddenly.

Throughout this terminal period, heart action appeared to remain normal, and did not cease until after complete respiratory failure. Upon autopsy, the heart was found to be in complete diastole; other than this, no gross abnormalities were evident.

The most obvious biochemical changes during this terminal period were of renal origin which, in turn, may have been secondary to anoxia. There was oliguria, a drop in the renal clearance of all substances measured, and a corresponding increase in their plasma concentration. There were, however, some metabolic manifestations apparently not related to renal failure. Thus, there were a decrease in the rate of urea formation and an acceleration of the rise in blood glucose. The latter cannot be ascribed to renal retention of glucose, since glycosuria did not occur in these animals. It may, however, have been secondary to anoxia. Some of the changes occurring in a typical animal are summarized in Table II. Changes in blood glucose and inorganic P concentration of the same animal are recorded in Fig. 2.

TABLE II

Biochemical Manifestations of Glycine Toxicity

Dog 6; weight, 15.9 kilos; glycine infusion rate, 2.9 mg. of N per kilo per minute; fluid infusion rate, 0.39 ml. per kilo per minute.

Period No.	Time	Plasma, mg. per cent		Urine flow, ml. per kilo per min.	Renal clearance, ml. per min.		
		α-Amino N	Urea N		Urea	α-Amino N	Inorganic P
	min.						
Control	61	4.55	10.1	0.01	25	1	4.6
2	46	29.5	13.2	0.096	39	22	25
3	42	39.4	16.7	0.41	47	39	37
4	51	49.2	18.2	0.40	43	36	19
5	47	58.6	19.8	0.44	43	34	16
6	38	69.0	23.8	0.42	36	28	8.6
Terminal	24	81.6	25.0	0.35	23	20	7.9

Several experiments were performed in an effort to protect the animal against glycine toxicity. Since glycine toxicity might have been due to a depletion of the body of essential metabolites rather than to direct toxicity of glycine or its oxidation products, three dogs were fed vitamin supplements for 1 week prior to the experiment, and a mixture of B vitamins and ascorbic acid was added to the infusion fluid. Two of these dogs were infused with glycine at rates of 0.85 and 1.0 mg. of N per kilo per minute; one of these expired after receiving 471 mg. of N per kilo and the other after

2140 mg. of N per kilo. The third dog received glycine at the rate of 2.5 mg. of N per kilo per minute and expired after the infusion of 610 mg. of N per kilo. In view of the variability of toxicity at the lower rates of glycine infusion, these results are not considered to show significant protection.

Six dogs were infused at varying rates with glycine solutions to which equal amounts of nitrogen as an enzymatic casein hydrolysate[1] were added. Of two dogs receiving this solution at about 1 mg. of glycine N per kilo per minute, one expired after 828 mg. of glycine N per kilo, and the other survived the infusion of 1750 mg. of glycine N per kilo. Two dogs receiving glycine-casein hydrolysate solution at approximately 2 mg. of glycine N per kilo per minute survived, respectively, 1670 and 1350 mg. of glycine N per kilo. When this solution was infused at rates of 4 mg. of glycine N per kilo per minute into two dogs, the experiment was complicated by an almost immediate marked negative water balance and profound dehydration of the animals. One expired after receiving 570 mg. of glycine N per kilo; the other survived 1302 mg. of glycine N per kilo. Thus, three of four dogs, receiving glycine-casein hydrolysate solutions at rates of 2 mg. of glycine N per kilo per minute or higher, survived the infusion. In view of the fact that among a total of eight dogs receiving, at comparable rates, glycine alone, or supplemented with glucose or vitamins or both, none survived a dose greater than 910 mg. of N per kilo (average lethal dose, 760 mg. of N per kilo); these experiments probably indicate significant protection.

Rate of Utilization of Glycine—Measurement of blood urea concentration alone, or urinary excretion alone, is not sufficient for the determination of total urea production and may lead to grossly erroneous results. Only Kiech and Luck (24), who measured changes in total carcass urea in rats, and Kirk (25), who calculated urea formation on the basis of total body water, have made valid measurements of total urea production from glycine in other than long term feeding experiments. Neither of these experiments was designed to measure maximum urea formation rates from glycine.

In the course of this study, it was established that the maximum rate of urea formation from glycine is approximately 0.8 mg. of N per kilo per minute. The relationship between glycine administration, the rate of disappearance of glycine from extracellular fluid, and urea formation is summarized in Table III. In Experiments 1 to 3, the glycine was infused first at a low rate and later at higher rates. However, it will be seen that the maximum rate of urea production from glycine could be attained at the low rates of administration, providing this rate exceeded 1 mg. of N per kilo per minute. Drastic increases in the rate of administration failed to elicit a rise in the rate of urea production; on the contrary there was some evidence of actual impairment of this process as indicated in Experiments 4, 5, and 10.

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[1] Amigen, Mead Johnson and Company.

Effects of Glycine Infusion upon Nitrogen Balance—Two conditions must be attained in order to evaluate the relationship between glycine metabolized and urea formed. First, the rate of urea production must be sufficiently high, so that variations in basal rate of urea formation would not lead to significant errors in evaluating the increment in urea formation due to glycine infusion. Second, the quantity of glycine metabolized must be accurately known. This necessitates a rate of glycine infusion which will not lead to excessive accumulation within cells, as the latter figure is not amenable to accurate measurement. It was found that an infusion rate in

TABLE III

Rate of Disappearance of Glycine N from Extracellular Fluid and Rate of Urea N Formation from Glycine at Varying Rates of Glycine Administration

The results are expressed in mg. of N per kilo per minute.

	Experiment No.	Glycine administration	Disappearance of glycine* from extracellular fluid	Urea formation† from glycine
Varying rates	1	0.91	0.85	0.41
		3.1	2.2	0.85
		5.5	2.0	0.75
	2	1.2	1.0	0.75
		3.1	1.6	0.75
		6.2	2.4	0.65
	3	2.2	1.1	0.40
		3.5	1.5	0.50
Low rates	11	1.03	0.97	0.95
	12	0.99	0.73	0.73
	13	0.91	0.86	0.87
Medium rates	6	2.9	1.5	0.65
	7	2.8	1.6	1.05
	8	3.1	1.9	1.15
	10	2.7	0.79	0.18
Rapid rates	4	7.9	1.8	0.45
	5	7.0	2.4	0.65

* Calculated as mg. of N administered minus (mg. of glycine N excreted + mg. of glycine N accumulated in extracellular fluid). This figure therefore represents that portion of the N administered which has entered cells, no matter what is its subsequent fate.

† Total urea N formation minus the average urea N formation of fasted controls.

the neighborhood of 1 mg. of N per kilo per minute best fulfilled these conditions. The results of these experiments are tabulated in Table IV. Only those experiments in which the plasma α-amino N level was relatively low were considered. Since, as found by previous workers (26, 27) and confirmed in these experiments (*cf.* Table VI), the increase in glycine concentration in skeletal muscle is considerably lower than the increment in plasma, while liver and kidney increments are the same or higher, a figure for total glycine accumulation in cells was calculated, empirically, as one-half the increment in plasma α-amino N concentration multiplied by the total cell volume of the animal. The total cell volume, in liters, was estimated as 70 per cent of the body weight expressed in kilos. Although the figure obtained in this manner is rather arbitrary at low levels of plasma α-amino

TABLE IV

Glycine Utilization and Urea Formation

The figures in parentheses refer to averages for the group.

Infusion	Experiment No.	Glycine N infused (1)	Glycine N utilized* (2)	Urea N formed (3)	Extra urea N, (3) − (2)	Extra urea N
		gm.	*gm.*	*gm.*	*gm.*	*mg. per kg. per min.*
Na_2SO_4, 1.5% (control)	DC-4	0	0	2.67	2.67	0.281
	DC-5	0	0	2.31	2.31	0.238
	DC-6	0	0	2.16	2.16	0.241
	DC-7	0	0	2.51	2.51	0.265
						(0.256)
Glycine, 1 mg. N per kilo per min.	11	11.84	10.66	13.43	2.77	0.242
	12	12.95	8.44	11.89	3.45	0.280
	13	15.07	14.72	20.12	5.40	0.277
	23V	14.88	13.90	19.70	5.80	0.320
						(0.280)
Glucose, 11 to 15 mg. per kilo per min. (control)	13G	0	0	1.30	1.30	0.116
	14G	0	0	1.22	1.22	0.128
	18G	0	0	1.16	1.16	0.095
	19G	0	0	1.35	1.35	0.157
						(0.124)
Glycine, 1 mg. N per kilo per min. + glucose, 11 to 15 mg. per kilo per min.	13GG	13.92	13.53	13.57	0.04	0
	14GG	6.45	5.87	5.97	0.10	0
	19GG	3.25	2.18	3.15	0.97	0.216
	20GG	6.04	5.90	6.56	0.66	0.096
	21GG	6.05	4.38	4.41	0.03	0
	22VG	2.62	1.91	2.19	0.28	0.095
						(0.068)

* (Glycine N administered) − (glycine N excreted + glycine N accumulated in extracellular fluid + glycine N accumulated within cells).

N, even gross errors in this figure lead to but small errors in the estimation of the total glycine metabolized.[2] When plasma α-amino N is greatly elevated, this method of calculation cannot, of course, be used.

It can be seen from Table IV that, when glycine was administered to a fasting animal, glycine administration had no effect upon the basal rate of urea synthesis. No sparing effect was noted, and no acceleration of protein "catabolism." When glucose was simultaneously infused in quantities sufficient to satisfy caloric requirements, the results were not as clear cut. Although some experiments indicate no effect upon the basal urea formation, some show a slight sparing action and others indicate that urea formation under these conditions is, within experimental error, equivalent to the amount of glycine metabolized. It can, therefore, be stated that glycine without glucose does not effect the basal rate of urea formation; in the presence of glucose, a sparing action has been observed.

Effect of Glycine upon Metabolism of Other Amino Acids—During the course of this study it was considered of interest to determine the effects of glycine upon the metabolism of other, non-protein, amino acids. To this end, analyses were performed to determine changes in plasma, urine, and tissue concentration of specific non-protein amino acids, as determined by microbiological techniques.

Over the course of several experiments, all of the essential amino acids with the exception of phenylalanine were determined in tungstate filtrates of plasma collected before and after glycine infusion. Although the number of determinations performed upon each animal was limited by the amount of plasma which could be safely withdrawn, determination of changes in plasma concentration of methionine, histidine, arginine, isoleucine, threonine, leucine, and tryptophan were performed on at least two separate occasions.

No significant or consistent changes in plasma concentration of these amino acids following glycine administration could be detected. Although, in several experiments, differences between initial and final samples of the order of 30 per cent were observed, these differences were not always present and, when present, occurred in either direction. Marked increases were observed in the excretion of other amino acids during the infusion of glycine. Table V indicates the excretion rates of certain amino acids during the control period, and the last complete infusion period before the appearance of toxic symptoms, of dogs receiving glycine sufficient to elevate their plasma α-amino N level to 40 to 65 per cent.

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[2] From the data in Table VI, at increments of plasma α-amino N concentration no greater than 15 mg. per cent, it is highly unlikely that the error in estimating cellular glycine would exceed a total of 500 mg. of N per 10 kilo dog, as this is the total increment estimated in such a dog's cells. In experiments of about 18 hours duration, such as these, this would entail a *maximum* error of (500 mg.)/(1000 minutes × 10 kilos) = 0.05 mg. of N per kilo per minute.

In control animals subjected to sodium chloride or sodium sulfate diuresis, the increase in excretion rate of specific amino acids noted in Table V was not observed. In one animal, which received glycine at the rate of 1 mg. of N per kilo per minute and which showed but slight elevation of plasma α-amino N concentration, there was no demonstrable increase in the rate of excretion of threonine or histidine. Another dog, receiving glycine at this same rate, but showing a plasma α-amino N concentration intermediate between the previous animal and the dogs listed in Table V, showed an increase in excretion of threonine and histidine one-half to one-third as great as the dogs listed in Table V.

TABLE V

Effect of Glycine upon Excretion of Essential Amino Acids

The results are expressed in mg. per minute; the figures in parentheses indicate the range. The rate of glycine administration was approximately 3 mg. of N per kilo per minute; the concentration of plasma α-amino N at the time of urine collection was 40 to 65 mg. per cent.

	Threonine (4 dogs)	Histidine (3 dogs)	Tryptophan (1 dog)	Isoleucine (1 dog)	Methionine (4 dogs)	Arginine (3 dogs)
Control excretion (1)	4.4 (2.7–6.2)	4.2 (2.8–5.0)	0.89	3.6	1.9 (0.9–2.7)	6.3 (5.6–7.4)
Excretion during glycine infusion (2)	294 (155–420)	227 (160–230)	14	52	22.2 (8.4–23)	27 (12–35)
Ratio, (2)/(1)	67 (50–89)	54 (46–59)	16	14	12 (8.5–24)	4.4 (2.0–5.9)

These findings indicate that the increased excretion and (in view of the relatively constant plasma concentration) increased clearance of the listed amino acids is a function of plasma glycine concentration. This is in accord with Pitts' concept (28, 29) that glycine inhibits the renal reabsorption of other amino acids by competition for a common reabsorptive mechanism.

The amino acids whose excretion rates are most markedly elevated during glycine administration are threonine and histidine, smaller increases being observed for methionine, tryptophan, and isoleucine. All of the above amino acids are among those for which Wright *et al.* (30) and Russo *et al.* (31) could demonstrate no reabsorptive T_M. Arginine, for which a T_M had been demonstrated (29, 30), shows the smallest increase in excretion rate. Although Beyer *et al.* (32) claimed that they could demonstrate no competition between the pairs glycine and arginine, and glycine and isoleucine, inspection of their data reveals increases in the renal clearance of these amino acids of the same order of magnitude as those observed in this study. However, in the presence of the very high percentage reabsorptions found by these workers, direct measurement of reabsorption rate is not sufficiently sensitive to demonstrate interference of one amino acid with the reabsorption of another. It is felt that the increases in excretion rate observed in this study, as well as the increases in clearance observed by Beyer *et al.* (32), are, in themselves, evidence for the interference of glycine with the renal reabsorption of other amino acids.

In this study, it was hoped that data on changes in concentration of specific non-protein amino acids in the livers of dogs and rats might indicate whether or not the infusion of glycine affected the metabolism of other amino acids. Although the data obtained occasionally appeared to indicate a slight fall in the concentration of specific non-protein amino acids in the livers of dogs, and a rise of similar magnitude in the livers of rats, wide fluctuations in values for both control and experimental animals rendered the data statistically inconclusive. If such phenomena do occur they are transitory and of relatively low magnitude. This would seem to be compatible with the failure of glycine to alter the basal rate of urea formation in fasted dogs.

The above series of experiments did, however, furnish valid data for the relationship between the total α-amino N content of rat livers and their content of threonine and methionine. These data are presented in Fig. 1.

The points in Fig. 1, each representing a separate rat liver, were obtained from rats receiving 5 per cent glucose, 1.5 per cent sodium sulfate, or 0.85 per cent sodium chloride intraperitoneally in six divided doses over a period of 2 hours. 7.5 ml. per 100 gm. of glucose solution and 16 ml. per 100 gm. of sodium chloride or sodium sulfate were administered. Since no differences were observed between groups of rats receiving each of the above solutions, no attempt to differentiate these groups is made in Fig. 1.

Fig. 1, besides indicating the distribution of liver concentrations of non-protein α-amino N, threonine, and methionine in rat livers, indicates a relative constancy of the ratio of these amino acids to the total free α-amino N over a wide range of α-amino N concentration. This in turn, suggests a constancy of the pattern of non-protein amino acids in rat liver.

Distribution of Glycine in Tissues—In the course of these experiments, a number of analyses for α-amino N content of tissues were performed for the purpose of ascertaining the tissue distribution of infused glycine. Since findings regarding the distribution of metabolites between cells and extracellular fluid can best be interpreted in terms of concentration in cellular and extracellular fluid water, the data in Table VI are presented in those terms.

From Table VI, it can be seen that, with the exception of Dogs 6 and 7, the increment of α-amino N in the cell water of liver was of the same order of magnitude as the increase in plasma concentration. The kidney increment, although somewhat smaller, was also of the same order. Heart muscle cells generally showed a still smaller increase, with skeletal muscle the smallest.

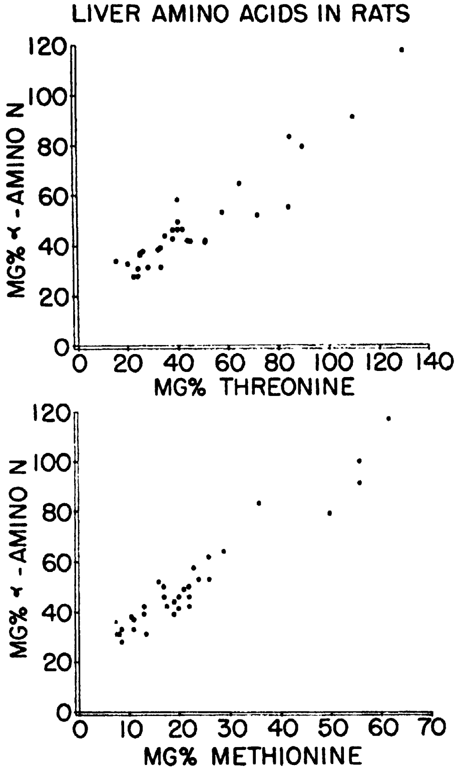

FIG. 1. Threonine and methionine concentrations of normal rat livers as a function of total α-amino acid N.

It should be noted that the values for increases in α-amino N concentration given in Table VI are approximations rather than exact figures, since they are determined by subtracting an *average* control figure from a figure experimentally obtained for the particular animal studied. Despite these limitations, it is felt that the conclusions reached in the previous paragraph are valid. Van Slyke and Meyer (26) and Friedberg and Greenberg (27) found that injected amino acids were concentrated in liver and kidney, and the conclusions reached by both groups remain valid when their data are recalculated in terms of concentration in cell water. In the series of experiments reported herein, most animals showed a liver and kidney cell increment approximately equivalent to the increase in plasma concentration; an increase greater than the plasma increment was observed in two instances. Van Slyke and Meyer (26) and Friedberg and Greenberg (27) collected tissue samples while plasma concentration was falling, whereas the tissue values found in this study were obtained in the presence of rising plasma α-amino N concentration. It is probable that this difference in experimental technique explains the discrepancies between these studies and those of the previous workers. The limited permeability of muscle cells for glycine previously reported (26, 27) is confirmed in these experiments. Data could not be found in the literature for the permeability of heart muscle cells to glycine, but the values shown in Table VI indicate a permeability greater than that of skeletal muscle cells but smaller than that of liver and kidney.

TABLE VI

Increases of α-Amino N in Cell Water of Various Tissues upon Administration of Glycine

The control values (mg. of α-amino N per 100 ml. of cell water) were as follows: liver (six dogs), average 88, range 73 to 114; kidney (2 dogs), average 130, range 127 to 134; heart (2 dogs), average 47, range 46 to 47; skeletal muscle (2 dogs), average 45, range 37 to 53.

Dog No.	Plasma amino N	α-Amino N per 100 ml. cell water minus average amino N in cell water of controls			
		Liver	Kidney	Heart	Skeletal muscle
	mg. per 100 ml. water	*mg.*	*mg.*	*mg.*	*mg.*
10	145	117			
5	133	136	136	83	52
4	124	119			
7	89	147	126	65	27
6	86	344	67	109	35
8	52	61	17	45	25
11	27	14			

The distribution of α-amino N between the water of plasma and erythrocytes is indicated in Table VII.

From the data in Table VII it may be seen that although control values for erythrocyte α-amino N, as previously noted by Hamilton and Van Slyke (6), are considerably in excess of plasma α-amino N concentration, the concentration at increased plasma glycine (as α-amino N) levels is essentially the same in plasma and red cell water, indicating free permeability. The lag period noted by Christensen *et al.* (33) was not detected in this study, probably because of the greater plasma glycine concentrations attained in these experiments.

Accessory Findings—In order to detect the formation of any possible nitrogenous intermediate other than urea formed during the metabolism of glycine, total N determinations were performed and "undetermined N" (total N minus urea N minus α-amino N) was calculated for plasma and urine samples of two dogs. No increases outside of experimental error were detected. Urine ammonia N, when measured, never totaled more than 5 per cent of the total urea N + ammonia N.

The excretion of extra glucose in phlorhizinized dogs following glycine administration has long been known (34), and the isotopic studies of Olsen, Hemingway, and Nier (35) have demonstrated the formation of liver glycogen from glcyine. However, little glycogen formation was noted in these studies, final liver concentrations ranging from 0.05 to 0.6 per cent. Shivering, which occurred during the course of many of these experiments, may have tended to vitiate the significance of the results obtained.

TABLE VII

α-Amino N in Red Cell and Plasma Water

The results are expressed in mg. per 100 ml. of water. The water content of plasma is 92 per cent; that of red cells, 65 per cent.

	Dog 5		Dog 6		Dog 7		Dog 8		Dog 9	
	Plasma H_2O	Cell H_2O	Plasma H_2O	Cell H_2O	Plasma H_2O	Cell H_2O	Plasma H_2O	Cell H_2O	Plasma H_2O	Cell H_2O
Initial	4.77	15.5	4.95	11.9	5.40	11.8	4.26	10.0	3.23	8.03
During glycine	111	106	64	64	45	39	32	31		
infusion	135	125	89	80	61	59	38	47	52	50
					68	66	47	51		
					91	93	47	56		
							56	59		

There is no agreement in the literature regarding the effect of glycine upon blood glucose concentration. Pollack (36) and Nord (37, 38) claimed hyperglycemic effects in rabbits and humans; Schenk (39, 40) states that a lowering of blood sugar was observed in these species, while Paasch (41) and Costa and Barone (42) could detect no changes in blood glucose concentration following the administration of glycine. Crandall and Cherry (43), however, in well controlled experiments, observed an increase in hepatic glucose output following the administration of glycine to dogs.

Fig. 2 shows that the rise in blood glucose concentration (observed in two typical experiments) was gradual at first but more rapid terminally.

Similar data were usually, but not invariably, found. Dog 2, for example, displayed a blood glucose level which failed to rise until shortly before death.

Although the data in Fig. 2 point to a definite hyperglycemic effect of glycine, this hyperglycemia is not necessarily attributable to gluconeogenesis from glycine. In view of the low liver glycogen values encountered, "glycogenolysis," perhaps secondary to anoxia, must be considered, particularly as a possible explanation for the precipitous terminal rise in blood sugar.

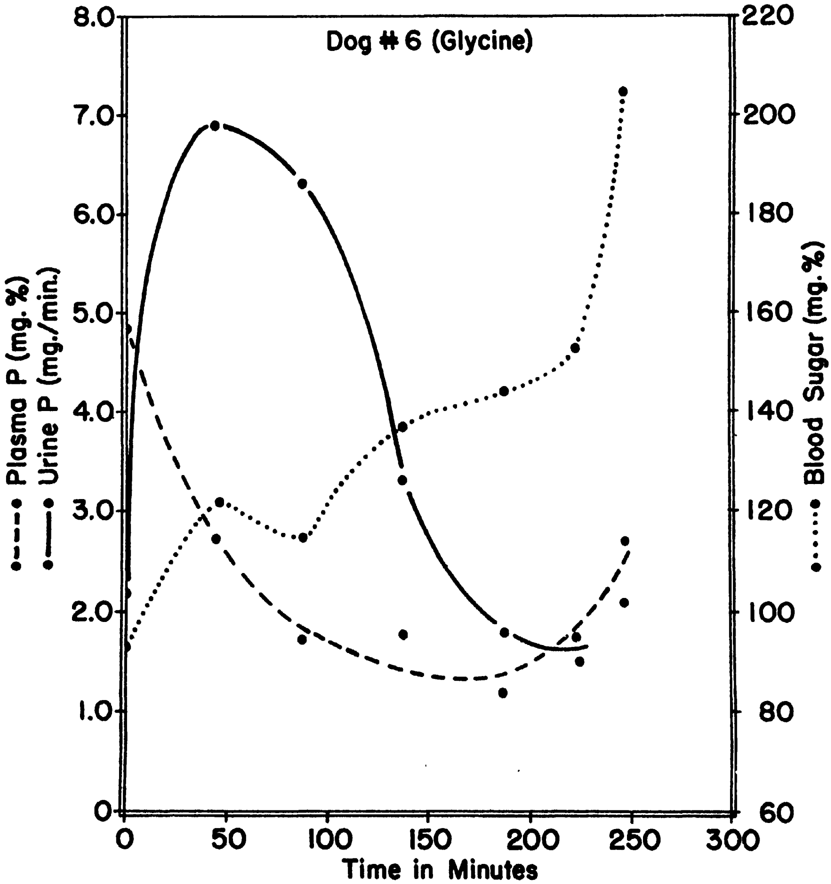

FIG. 2. Blood glucose, plasma P, and urine P in a dog given glycine intravenously at the rate of 2.9 mg. of N per kilo per minute.

Fig. 2 also illustrates the dramatic drop in plasma inorganic P concentration and rise in urine inorganic P excretion during the course of glycine administration. However, similar changes were observed in dogs subjected to sodium chloride and sodium sulfate diuresis and this effect cannot, therefore, be attributed to either glycine administration or hyperglycemia. Additional data obtained in these studies indicate that neither plasma CO_2-combining capacity nor chloride concentration was consistently affected by glycine administration.

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SUMMARY

1. Techniques have been devised and conditions established for the study of the metabolism and utilization of parenterally administered amino acids. Glycine was selected for this study.

2. Glycine, administered by continuous intravenous infusion, is lethal to dogs in dosages ranging from about 0.4 to 1 gm. of N per kilo. The toxicity of glycine appears to correlate best with total dosage, although there are indications that somewhat larger quantities may be tolerated at slower infusion rates. The mode of death and concomitant findings are described.

3. In the dog, the maximum rate of urea formation from glycine, including both excretion and accumulation within body water, is about 0.8 mg. of N per kilo per minute.

4. When glycine is administered over periods of 15 to 18 hours to fasting dogs, there is no evidence of either a sparing or an accelerating effect upon protein catabolism. However, when glycine is administered together with sufficient glucose to satisfy caloric requirements, a sparing action of glycine is observed.

5. The rate of excretion of the six essential amino acids which were measured was markedly increased during glycine infusion. The magnitude of this increase is dependent upon plasma glycine concentration. The administration of glycine causes no consistent changes in plasma concentration of free essential amino acids.

6. The concentration of non-protein threonine and methionine in the livers of rats varies directly with the total free α-amino N, indicating a constancy of free amino acid pattern.

7. Erythrocytes appear to be freely permeable to infused glycine. In most cases, liver and kidney cells appear to increase in α-amino N content by an increment approximately equal to the rise in α-amino N. However, some significantly greater increases have been observed. The rise in heart

muscle cell α-amino N is smaller than that in liver or kidney; the smallest increases were observed in skeletal muscle cells.

8. During glycine infusion, plasma chloride, CO_2-combining capacity, and plasma and urine "undetermined N" remain within normal limits.

9. A markedly decreased plasma inorganic P concentration, accompanied by an increased urinary P excretion, was observed in dogs receiving glycine, sodium chloride, or sodium sulfate infusions.

10. Blood glucose concentration increases during glycine administration, the increase being most marked terminally. Liver glycogen concentration is low.

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BIOCHEMICAL STUDIES OF VIRUS REPRODUCTION

I. PURIFICATION AND PROPERTIES OF ESCHERICHIA COLI BACTERIOPHAGE T_6*

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Bacteriophages possess the fundamental properties of viruses, yet exhibit ease of assay, handling, and culture, and thus present suitable systems for isotope tracer studies of the mode of virus reproduction and protein synthesis. In an investigation of this problem we have employed the *Escherichia coli* bacteriophage T_6 system. This communication outlines methods for the growth and isolation of this phage and describes the properties and physicochemical characterization of the purified virus. A following paper[1] discusses the chemical composition of bacteriophage T_6. Later articles will cover the application of isotope indicators to the study of the biological precursors of virus nucleic acid and protein. A preliminary report on the origin of virus phosphorus has already been published (2).

Numerous biological studies on the bacteriophages attacking *E. coli* have recently appeared. The seven types of coliphages most commonly studied (T_1, T_2, ... T_7) were first described by Demerec and Fano (3). Their biological properties and their differentiation on the basis of plaque type, morphology, size, antigenic structure, and host range have been reviewed by Delbrück (4, 5). Although electron micrographs of several of these coliphages have been published (6–8), physicochemical characterization has thus far been meager. Only bacteriophage T_2 has previously been isolated in quantity and characterized in the ultracentrifuge (8, 9), and electrophoretic diagrams have not yet been published for any of the bacterial viruses.

EXPERIMENTAL

Growth and Purification of Bacteriophage T_6

Materials and Methods—The *E. coli* bacteriophage studied in this investigation is the wild type, strain T_6r^+, which produces small plaques

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[1] Putnam, F. W., and Kozloff, L. M., in preparation.

and causes lysis inhibition upon multiple infection of the host bacterium. Antigenically and morphologically it is closely related to coliphage T_2 isolated and studied by Hook *et al.* (8–10) and by Cohen (11, 12). In early experiments culture in nutrient broth medium was carried out with a strain of T_6 and its sensitive host *E. coli*, strain B, both received from Dr. M. Delbrück, California Institute of Technology.[2] When it was found that this phage could not be grown in quantity in synthetic medium by the procedure of single infection (*cf. below*), another strain of T_6 phage was employed for culture both in nutrient broth and synthetic medium. The latter strain, which was obtained from Dr. S. Luria, Indiana University, was known to yield high titers in synthetic medium under conditions of multiple infection, and had been selected for freedom from the requirement for tryptophan as an adsorption cofactor. Both strains of T_6 were originally derived from a common stock described by Demerec and Fano (3). No significant differences have been found in the two strains with regard to physicochemical properties by ourselves, or with regard to biological characteristics by Dr. Luria (personal communication). As described in the text, success in culture in synthetic medium is attributed chiefly to the procedure of infection rather than to the strain of phage employed. Phage type was verified by reference to plaque form, latent period, and host range. Most of the data in this paper refer to purified concentrates of the strain received from Luria.

Phage assay was performed by the plaque count method of Ellis and Delbrück (13) as modified by Hershey *et al.* (14). After serial dilution of the sample in sterile broth, duplicate plates of the final dilution were left overnight at room temperature and the plaques counted the next day. The host bacteria were maintained on a 1 per cent agar slant with daily transfers. The complete medium consisted of 0.8 per cent Difco-nutrient broth with 0.5 per cent NaCl added, final pH about 6.8. The agar was composed of the above nutrient broth with 1 per cent Difco-Bacto-agar added. The synthetic medium, essentially that suggested by Luria,[3] was made up in distilled water by addition of boiled neutralized lactic acid and stock solutions of salts. Final percentages of salts were sodium lactate 1.1, KH_2PO_4 0.15, Na_2HPO_4 0.35, NH_4Cl 0.01 and $MgSO_4$ 0.001.

Bacteriophage T_6 may be grown on *E. coli* cultured either in broth or in the synthetic medium, but in the latter medium the yield of phage varies greatly with the manner of infection. For *single infection* experiments a subculture of bacteria is infected with one virus particle per 400 to 600 bacteria 5 or 10 minutes prior to inoculation of the medium. In this method, uninfected bacteria are in great excess initially, and statistically only one virus particle is adsorbed per infected cell. Many generations of phage result. In *multiple infection* experiments the final actively growing bacterial culture is infected with phage, so that theoretically each cell adsorbs more than one particle. Under the latter conditions lysis is inhibited and the liberation of phage is delayed (15). Turbidity measurements remained nearly constant under conditions of multiple infection, but colony counts revealed that the number of viable bacteria fell steadily from 10^8 cells per ml. to 10^5 cells per ml. or less in the first few hours of incubation. These results and other unpublished data indicate that multiple infection accompanied by lysis inhibition largely yields a single generation of virus.

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[2] In preliminary experiments coliphage T_1, differing serologically, morphologically, and in plaque form from T_6, was grown on the host both in broth and in synthetic medium. This smaller phage is less readily concentrated in the supercentrifuge and angle centrifuge and is markedly unstable upon high dilution in saline.

[3] Luria, S., private communication.

Isolation from Nutrient Broth Lysates—Single and multiple infection methods were used with equal success in propagation of the bacteriophage in nutrient broth, but the large amount of infecting virus required in the latter procedure discouraged its use.

Nutrient broth in 10 to 24 liter lots was inoculated at a volume ratio of 1:50 with an 18 hour subculture of bacteria, infected as described above for single infection. After 8 hours incubation with aeration at 37°, the cultures were pooled and stored in the cold. It was found that the course of phage growth was almost logarithmic up to 6 to 8 hours, the final titer approaching 10^{10} phage per ml. It was not necessary to store fresh broth lysates in the cold to aid in the elimination of mucoid material, as reported by Hook *et al.* for bacteriophage T_2 (8).

The bacterial débris could be removed from the lysate either by filtration through 10 inch Mandler candles of medium porosity or by clarification in the Sharples supercentrifuge at 40,000 R.P.M. (39,000*g* at the bowl periphery) and a rate of flow of 10 liters per hour. Filtration appeared to be more efficient. The filtered or clarified phage was then concentrated by centrifugation at 56,000*g* in the virus concentration bowl of the Sharples (Presurtite laboratory model) supercentrifuge by a method similar to that described by Hook *et al.* (8). The pooled opalescent Sharples concentrate of bacteriophage was purified by differential centrifugation in the Sorvall high speed angle centrifuge rather than in the ultracentrifuge. One step at 2000*g* for 15 minutes was employed to remove particulate matter, and another at 18,000*g* for 2 hours to sediment the virus. All centrifugation was carried out in the cold.

Purification by two such cycles of differential centrifugation usually sufficed to give concentrates of approximately constant infectivity (infectivity being defined as gm. of N per phage). The arithmetic average of the infectivity of more than ten concentrates of broth phage T_6 was $10^{-15.86}$ gm. of N per phage, with extreme values of $10^{-15.75}$ to $10^{-16.02}$. These values compare closely with the average figure ($10^{-15.88}$) and the range given for T_2 phage concentrated in the ultracentrifuge (8). The progress of purification is shown by representative data given in Table I for a preparation later characterized in the electron microscope, ultracentrifuge, and the electrophoresis apparatus. It may be seen that on a nitrogen basis a purification of 1000-fold is achieved. In this instance, the recovery of phage from the filtered lysate amounts to about 47 per cent and corresponds to a yield of about 3.5 mg. of phage per liter of culture. In other experiments, yields up to 8 mg. of phage per liter of broth culture have been obtained with average recoveries of 60 per cent. A higher percentage of the virus may be recovered by passing the lysate through the supercentrifuge at a lower rate of flow than that employed in this experiment (2 liters per hour).

TABLE I

*Isolation of T_6 Bacteriophage from Broth Lysates**

Material	Volume	Titer	N content	Infectivity
	ml.	*phage per ml.*	*mg. per ml.*	*gm. N per phage*
Filtered lysate	9000	7.4×10^{9}	1.16	$10^{-12.80}$
Sharples effluent	8620	1.8×10^{8}	1.22	$10^{-11.17}$
" concentrate	95	3.0×10^{11}	0.08	$10^{-15.57}$
Angle supernatant (1st cycle)	93	3.1×10^{10}	0.034	$10^{-14.96}$
" concentrate (" ")	15.2	1.7×10^{12}	0.35	$10^{-15.69}$
" " (2nd ")	14.5	2.16×10^{12}	0.276	$10^{-15.89}$
" " (3rd ")		1.43×10^{12}	0.202	$10^{-15.85}$

* Nutrient broth inoculated 1:50 with singly infected bacteria (bacteria to phage = 400:1) (Lot XI). Incubation for 8 hours at 37° with aeration. Sharples centrifugation at 48,000 R.P.M. (56,000*g* at the bowl periphery) at a constant rate of flow of 2 liters per hour.

Isolation from Synthetic Medium—In synthetic (lactate) medium the growth of the host bacterium is much slower. Accordingly, lysates of high phage titer were never obtained from these cultures by the procedure of single infection, even with prolonged incubation. This result is contrary to that observed with T_2 bacteriophage in synthetic medium containing glucose (8). In experiments to be described in detail elsewhere[4] the growth of T_6 phage in lactate or glucose media was studied by the one-step growth curve technique of Delbrück and Luria (16), with broth as a control. These experiments demonstrated that the yield of virus particles per infected bacterium in synthetic medium fell to one-tenth that observed in broth. A search for growth factors revealed that casein hydrolysate, yeast extract, and a small addition of broth gave marked stimulation, while several amino acids, purines, and vitamins increased phage growth somewhat. However, no single specific factor was found and at the suggestion of Dr. Luria the procedure of multiple infection, already described, was investigated.

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[4] Neil, J., Kozloff, L. M., and Putnam, F. W., in preparation.

After study of the optimum conditions for growth of the phage by the above method, it was found that large lots of actively growing bacteria at a concentration of 1 or 2 × 10^8 cells per ml., when infected with phage at a ratio of 1 to 5 virus particles per organism, gave lysates with titers up to 2 × 10^{10} phage per ml., upon overnight incubation with aeration at 37°. Titers up to 4 × 10^{10} phage per ml. were sometimes obtained after 24 hours incubation with no apparent adverse effect of prolonged incubation. The lysates contained visible suspended particulate matter which was readily removed by filtration on Mandler candles. With the procedure of multiple infection, synthetic medium cultures proved to be a better source for the production of T_6 phage than did broth. In one instance 24 liters of culture yielded 442 ml. of crude Sharples concentrate containing 2.1 × 10^{12} phage per ml., corresponding to a yield of 47 mg. of phage per liter of culture. Electrophoretic analysis (see below) of the crude concentrate indicated the absence of free nucleic acid and revealed the presence of a major boundary with the mobility of phage and of a minor boundary representing a trace of impurity. In accord with this result, the infectivity of clarified Sharples concentrates of the synthetic medium phage in several instances ranged from $10^{-15.8}$ to $10^{-15.9}$.

However, on standing in the cold, synthetic medium concentrates undergo rapid loss of activity as compared to broth phage (see below), and purification by differential centrifugation leads to disruption of much of the virus with liberation of nucleic acid. In addition to the limpid pellet and clear supernatant found in the high speed sedimentation of broth phage, the synthetic medium concentrates contained an intermediate layer of insoluble, viscous material, presumably nucleic acid. As indicated later, all our concentrates from synthetic medium have revealed at least a trace of unbound nucleic acid upon electrophoretic analysis.

Ethanol Fractionation—Procedures for protein fractionation were investigated in an attempt to avert the loss of activity incurred on differential centrifugation of synthetic medium phage. Addition in the cold of saturated $(NH_4)_2SO_4$ to a final concentration of 2 M at pH 5.4 resulted in nearly quantitative precipitation of the phage, but with much loss of activity. However, graded dialysis against buffered ethanol solutions at −1° to −5° afforded a ready means for precipitation of the virus. This procedure involves successive dialysis of the phage against ethanol solutions of the following concentrations: 8, 12, 16, 20, and 30 per cent. Each liter of dialysis solution contained 15 ml. of 1 N sodium acetate buffer, pH 5.4, the calculated amount of 95 per cent ethanol, and 0.9 per cent saline to volume. The precipitate obtained with 16 per cent alcohol is impure and is usually discarded. After centrifugation at 2000*g* for 15 minutes, the precipitate at higher concentrations of ethanol readily dissolves in saline to give a concentrated opalescent solution with high infectivity. Although most of the phage is precipitated at 20 per cent ethanol, as seen in Table II, the precipitate at 30 per cent alcohol is more readily centrifuged.

TABLE II

Ethanol Concentration of Synthetic Medium Bacteriophage T_6 at pH 5.4

Precipitation	Material	Ethanol concentration	Titer	Volume	Total phage	N content	Infectivity
		per cent	*phage per ml.*	*ml.*	*10^{12}*	*mg. per ml.*	*gm. N per phage*
1st	Sharples concentrate*		6.4×10^{11}	125	80	0.070	$10^{-15.8}$
	Supernatant	30	7.2×10^{10}	125	9	0.023	$10^{-15.5}$
	Ppt.	30	3.8×10^{12}	17	66	0.408	$10^{-15.97}$
2nd	"	16	5.6×10^{11}	5	2.8	0.105	$10^{-15.78}$
	Supernatant	20	8.1×10^{10}	17	1.4	0.025	$10^{-15.52}$
	Ppt.†	20	3.0×10^{12}	10.6	31.8	0.495	$10^{-15.78}$
3rd	"	16	6.2×10^{11}	5	3.1	0.099	$10^{-15.8}$
	Supernatant	30	8.7×10^{10}	20	1.7	0.024	$10^{-15.68}$
	Ppt.‡	30	2.5×10^{12}	10	25	0.201	$10^{-16.1}$
4th	Supernatant	30	8.2×10^{10}	16	1.3		
	Ppt.§	30	1.44×10^{12}	8.5	12.3	0.190	$10^{-15.92}$

* For electrophoretic diagrams at pH 6.10, see Fig. 3, *C*.

† For electrophoretic diagrams at pH 6.40 and 7.60, see Fig. 3, *D* and *E*.

‡ For electrophoretic diagrams at pH 8.58, see Fig. 3, *F*.

§ For electrophoretic diagrams at pH 5.2, see Fig. 3, *G*.

The dialyses are carried out in the cold, just above the freezing point of the mixture, to avert inactivation which occurs slowly at 4° but rapidly at 30° in 20 per cent ethanol. The pH is also critical; at pH 7 no phage is precipitated at a concentration of 30 per cent ethanol, the yield increasing as the pH is adjusted closer to the acidic limit of the stability region.[5]

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[5] At pH 5.1 the phage is largely precipitated by 12 per cent ethanol.

Further investigation of the optimum conditions for precipitation is under way.[6]

The progress of purification by alcohol fractionation was followed in four preparations by phage assay, infectivity measurements, and electrophoretic analysis. The electrophoretic diagrams, to be discussed later, are in accord with the activity data given in Table II for ethanol fractionation, and also with the data on differential centrifugation given previously in Table I. All the results indicate that by far the greater part of both concentration and purification is accomplished by the preliminary centrifugation in the supercentrifuge.

Properties of Purified Bacteriophage T_6

Suspensions of purified bacteriophage are bluish in reflected light at concentrations of 5×10^{10} particles per ml., opalescent at 10^{11} phage per ml., and milky at 10^{12} per ml. Over the range, 4×10^{10} to 6.5×10^{11} particles per ml., the activity of electrophoretically homogeneous samples is linearly related to light absorption measured in the Coleman spectrophotometer (model 6A) at 400 mμ.

The complete absorption spectrum of synthetic medium bacteriophage, concentrated by the ethanol precipitation and purified by electrophoretic separation at pH 5.1, was measured in the Beckman quartz spectrophotometer (model DU). The spectrum is given in Fig. 1. From the inset diagram of Fig. 1 it can be seen that in the near infra-red and in the visible range the spectrum shows a steady increase in apparent light absorption as the wave-length decreases. When the optical density is plotted against the 4th power of the wave-length, a nearly linear relationship is observed in the visible region. This result, which is in accord with the Rayleigh-Mie equation (17), indicates that the bluish appearance of the phage is due solely to molecular light scattering.

In the ultraviolet region, however, the phage exhibits intense absorption, necessitating the use of a different scale for the ordinate in Fig. 1. The spectrum is that typical of nucleic acids, with a sharp maximum at 262 mμ and a minimum at 240 mμ. Since the bacteriophage is morphologically and chemically complex and its phosphorus is distributed among several compounds,[1] the results are expressed, not as the "molecular" extinction coefficient or the atomic extinction coefficient with regard to P, but as the specific absorption coefficient, $K = (\log 1/T)/C$, where $C =$ gm. of P per liter, and T equals the per cent transmission.

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[6] Other methods of purification still being investigated include acid precipitation at pH 4.2, followed by rapid dissolution of the precipitate in buffered saline. In one experiment with synthetic medium phage the activity was recovered quantitatively and the product had an infectivity of $10^{-15.94}$. Electrophoretic analysis revealed that the preparation contained about 2 per cent free nucleic acid as the only impurity. In a second experiment, on study both in the electrophoresis apparatus and in the analytical ultracentrifuge, acid-precipitated phage exhibited a single major boundary representing the phage, and a minor boundary identified in electrophoresis, at least, as DNA.

Stability—Phage from either broth or synthetic medium cultures is soluble in physiological saline after purification by differential centrifugation, but dissolves more readily when concentrated by ethanol precipitation. The phage precipitates on dialysis against distilled water; lyophilization renders it insoluble and inactive. The virus is disintegrated when held at 100° for 1 minute and inactivated if heated at 55° for 30 minutes.

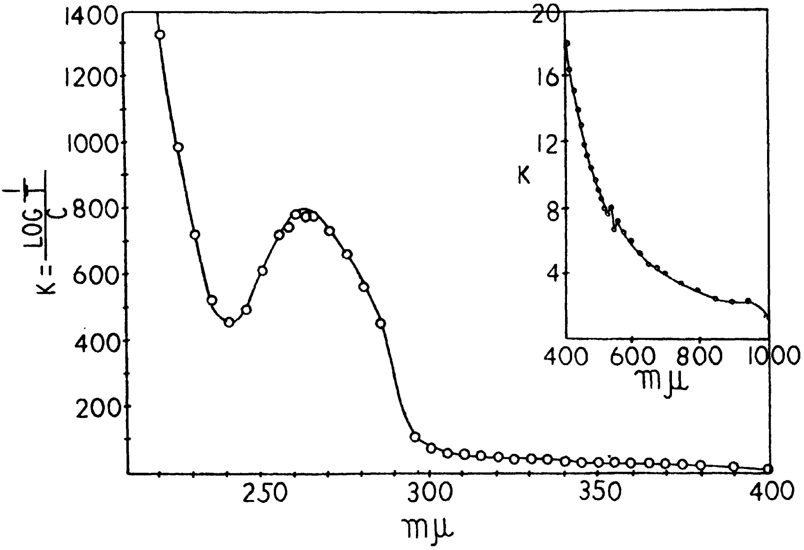

FIG. 1. Complete absorption spectrum of synthetic medium *Escherichia coli* bacteriophage T_6 concentrated by ethanol precipitation and purified by electrophoretic separation. The specific absorption coefficient, K, is defined in the text. In the inset, graph K is given in the same units for visible and infra-red light as for ultraviolet light in the main figure.

The pH stability range for purified broth phage, as determined by assay at intervals over a period of 9 weeks, is about pH 4.9 to 8.6. Over this time interval, the titer of broth phage drops slowly, but the synthetic medium phage is inactivated quite rapidly with the formation of a stringy sediment. Below pH 4.2, the phage precipitates immediately, and also precipitates on standing at pH 4.6 to 4.8. The solution retains its characteristic opalescence throughout the stability range but clears and becomes viscous at about pH 10. Nucleic acid is liberated, as can be shown by precipitation in 4 volumes of cold ethanol.

The relationship of solubility to infectivity is illustrated in Fig. 2, which shows the pH stability range for synthetic medium phage (initial titer of 6.5×10^{11} phage per ml.) after 72 hours at 4° in acetate-veronal buffers of 0.05 ionic strength or glycine buffers of 0.1 ionic strength. The left-hand ordinate represents the per cent of initial activity remaining; the right-hand ordinate gives the optical density of the supernatant solution as measured in the Coleman spectrophotometer at 400 mμ. The close coincidence of the stability range for infectivity and of the solubility region is noteworthy. While nucleic acid is split off in the alkaline region, the phage precipitate obtained at pH 4 retains activity for some time.

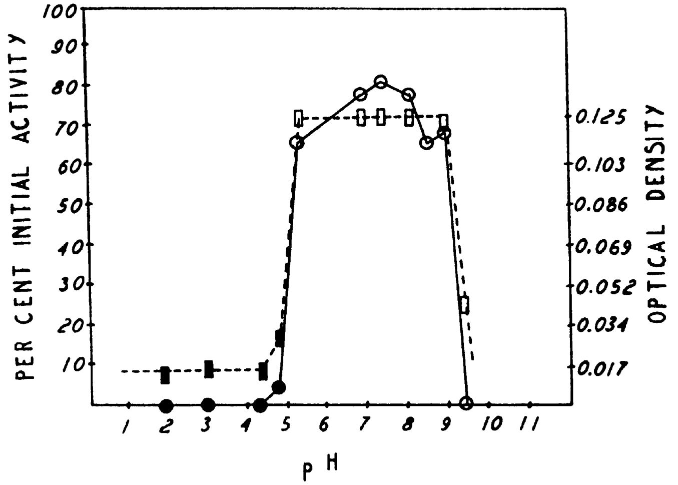

FIG. 2. pH stability and solubility range of *Escherichia coli* bacteriophage T_6 isolated from synthetic medium. The left-hand ordinate corresponding to the solid line (○) gives the per cent initial activity remaining after 72 hours at 4°. The right-hand ordinate referring to the broken line (□) gives the optical density of the supernatant solution measured at 400 mμ. The solid black points on either graph indicate the presence of a precipitate.

As indicated previously,[6] advantage of this fact may be taken in the purification of bacteriophage.

Phage biologically labeled with P^{32} has been obtained with a specific activity of 760 counts per minute per γ of P without alteration of normal infectivity;[7] but, like other coliphages, T_6 is rapidly inactivated by ultraviolet irradiation. Activity of the virus is unaffected by cacodylate or veronal buffer up to pH 8.6, or by incubation with trypsin. Reduction with cysteine or thioglycolic acid inactivates the virus, but the effect is partially reversible on oxidation with cystine. Phage infectivity is not affected by 0.02 M fluoride but a 1:1000 dilution of the cationic detergent, benzyldodecyldimethylammonium chloride, abolishes the activity. At the same concentration, cetyltrimethylammonium bromide and the anionic detergent, sodium dodecyl sulfate, are less effective. However, at pH 8, sodium dodecyl sulfate splits off nucleic acid from the virus at a phage to detergent weight concentration ratio of unity.

Physicochemical Analysis

Electrophoretic Homogeneity—Electrophoretic analysis was performed at 0.6° or at 2° in the standard Klett electrophoresis apparatus equipped with the Longsworth schlieren scanning system (18).[8] Because of the high opalescence of the solutions and the limited amounts of purified virus, phage concentrations of about 0.2 per cent were at first employed. For concentrations of 0.4 per cent studied in later work it was necessary to resort to infra-red light photography in order to depict the solution side of the boundary. A tungsten light source together with Wratten filter No. 89 or 89A and infra-red sensitized film or plates served for this purpose.

The broth bacteriophage characterized in the electron microscope and in the ultracentrifuge moved as a single diffuse boundary in the electrophoresis apparatus at a concentration of 0.2 per cent at pH 6.69 (Experiment 1, Table III). Another batch of broth phage exhibited 5 to 10 per cent of a fast moving component with mobility of -16.1×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$ at pH 7.60 (Experiment 2, Table III). Prior to chemical identification[1] the fast component was assumed to be free nucleic acid on the basis of its mobility (*cf.* Experiments 13 and 14, Table III). Some preparations of broth phage migrated without evidence of free nucleic acid or other impurities over the pH range studied, pH 5.1 to 7.6 (Experiments 3 to 6, Table III; Fig. 3, *A* and *B*). Other preparations of broth phage, generally of lower infectivity ($10^{-15.74}$), exhibited two components with closely similar mobilities over the pH range of study (*e.g.*, 5.1 and 5.9×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$ at pH 6.20). The more slowly moving boundary represented the major component.[9] Though the principal boundary separated clearly, it remained diffuse and skewed and could not be isolated in the separation cell. In a single preliminary experiment, broth phage, showing two principal boundaries and a trace of nucleic acid on electrophoresis, was studied simultaneously in the analytical ultracentrifuge

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[8] Some of the electrophoretic analyses were performed by Mr. Eugene Goldwasser. The electrophoresis apparatus and the electrically driven analytical ultracentrifuge were made available through the courtesy of Dr. E. S. G. Barron, Department of Medicine, University of Chicago.

[9] A concentrate of coliphage T_1, which forms large plaques, was studied in electrophoresis at pH 7.6. Two boundaries were observed with mobilities of 8.5 and 10.7×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$, neither corresponding to the mobility of T_6 phage at this reaction.

On sedimentation three boundaries were obtained, one sedimenting slowly and presumably representing nucleic acid, and two principal boundaries with S_{20} = 794 and 1009 Svedberg units. This result suggests that the double boundary phenomenon in the ultracentrifuge may have an analogue in electrophoresis.

TABLE III

Electrophoretic Analysis of Escherichia coli Bacteriophage T_6*

B signifies nutrient broth medium, L synthetic (lactate) medium.

Experiment No.	Fig. No.	Source	Infectivity†	Concentration	pH	Time	Electrophoretic mobility,‡ 10^{-5} sq. cm. volt^{-1} sec.$^{-1}$			
							Bacteriophage		Nucleic acid	
							Ascending	Descending	Ascending	Descending
			gm. N per T_6	*mg. N per ml.*		*sec.*				
1		B	$10^{-15.89}$	0.276	6.69	18,000	−6.04	−5.64		
2		"	$10^{-15.86}$	0.247	7.60	7,200	−7.44	−7.10	−17.3	−16.1
3	3A	"	$10^{-16.03}$	0.282	7.60	23,910	−6.84	−6.55		
4	3B	"	$10^{-15.86}$	0.35	6.63	14,400	−6.58	−6.35		
5		"	$10^{-15.78}$		5.78	17,520	−4.36	−4.36		
6		"			5.10	18,000	−3.77	−3.55		
7		L	$10^{-15.77}$	0.259	6.40	10,320	−7.19	−6.15	−18.0	−17.9
8	3C	"			6.10	14,400	−4.94	−4.91		
9	3D	"	$10^{-15.78}$	0.495	6.40	12,500	−6.01	−5.97	−17.9	−18.4
10	3E	"	$10^{-15.78}$		7.60	16,800	−6.20	−5.94	−17.4	−16.8
11	3F	"	$10^{-16.1}$	0.201	8.58	8,200	−7.37	−7.34	−16.5	−17.5
12	3G	"	$10^{-15.92}$	0.190	5.18	3,100	−4.01	−3.62	−14.5	−17.5
13		Thymus	DNA	0.910	7.60	6,780			−19.3	−17.6
14		"	"	0.710	5.50	5,400			−19.2	−16.6
15§		Yeast	RNA		5.34					−13.1

* Ionic strength = 0.1 in all instances except pH 6.69 (Experiment 1). Composition of buffers as follows: pH 6.69, 0.1 M NaCl-0.025 M Na_2HPO_4-0.025 M KH_2PO_4; pH 7.60, 0.07 M NaCl-0.03 M Na veronal; pH 5.78 to 6.63, 0.08 M NaCl-0.02 M Na cacodylate; pH 5.10 to 5.5, 0.1 M Na acetate; pH 8.58, 0.1 M Na veronal.

† The infectivity of phage recovered from the electrophoresis cell was undiminished in the range, pH 5.18 to 8.58. A small drop was experienced at pH 5.1.

‡ Temperature 2°. The voltage gradient was less than 4 volts per cm. in all instances and about 1.2 volts per cm. in most experiments with phage.

§ Data of Longsworth and MacInnes (19).

Typical electrophoretic patterns for broth phage showing no evidence of freely migrating nucleic acid at pH 7.60 and 6.63 are given in Fig. 3, *A* and *B*. In these cases, as in all instances, the boundaries are diffuse and skewed. As shown in Fig. 4, boundary sharpness was not restored by reversal of the electric field with migration continued until the origin

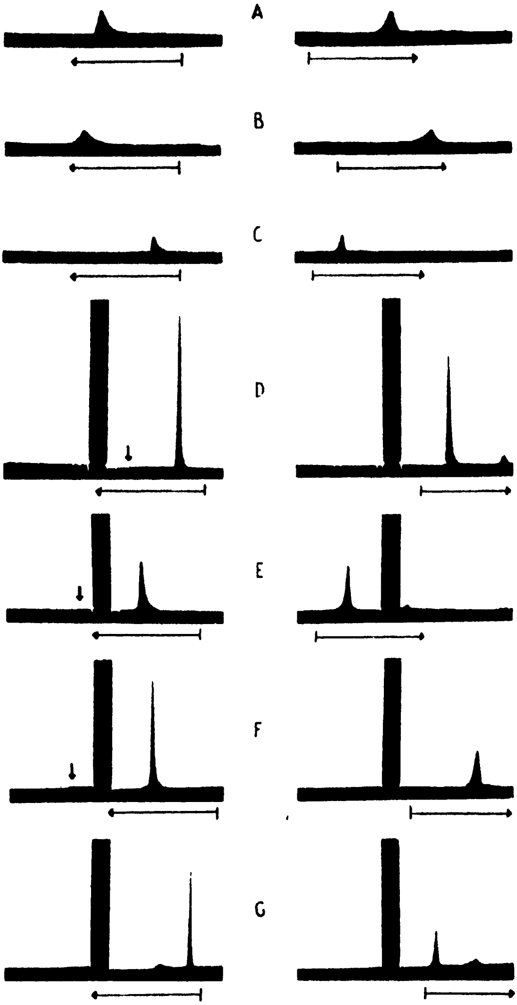

FIG. 3. Electrophoretic analysis of *Escherichia coli* bacteriophage T_6 isolated from broth or synthetic medium lysates. The source of the phage, its infectivity and concentration, the pH, and the conditions of electrophoresis are given in Table III for experiments corresponding to Fig. 3, *A* to *G*. The horizontal arrows indicate the direction of migration, left diagram ascending, right descending. The tail of the arrow signifies the position of the starting boundary. The vertical arrows denote traces of fast moving components; the vertical bar in Experiments *D* to *G* is the image of the ground glass sliding section of the separation cell used in these experiments.

was again attained. The factors contributing to this effect are considered in the "Discussion."

While broth bacteriophage gave a single boundary over the range pH 5.1 to 7.6, in some experiments it was impossible to exclude the presence of minute amounts of freely migrating nucleic acid because of the low concentrations employed. Because of the acid precipitation of phage already mentioned, it was not feasible to conduct electrophoretic analysis below pH 5.1, and even at this reaction the ascending limb became turbid. In all experiments reported in Table III the infectivity of preparations recovered from the electrophoresis cell was undiminished, except for a small drop at pH 5.1.

From our best lot of synthetic medium phage a crude Sharples concentrate containing 2.1 × 10^{12} phage per ml. was obtained. This was submitted to electrophoresis at pH 6.1 without further purification, and the typical skewed patterns given in Fig. 3, *C* were observed. The crude concentrate appeared to consist predominantly of one component. No free nucleic acid was detectable, but there appeared to be a trace of somewhat faster moving material too slight to be observable in the figure. On standing several months, the activity of this crude concentrate dropped to 6.5 × 10^{11} phage per ml. Ethanol purification by two precipitations at 30 per cent alcohol, pH 5.4 (*cf.* Table II), yielded a preparation which contained about 10 per cent free nucleic acid at pH 6.40, according to the electrophoretic patterns for the descending limb (Fig. 3, *D*). However, on the ascending side the fast moving component, indicated by the vertical arrow in Fig. 3, *D*, represented only 2.5 per cent of the total area. Other patterns given in Fig. 3 illustrate the same phenomenon of an apparently smaller amount of free nucleic acid in the ascending diagram. A similar phenomenon has been observed in electrophoretic studies of the

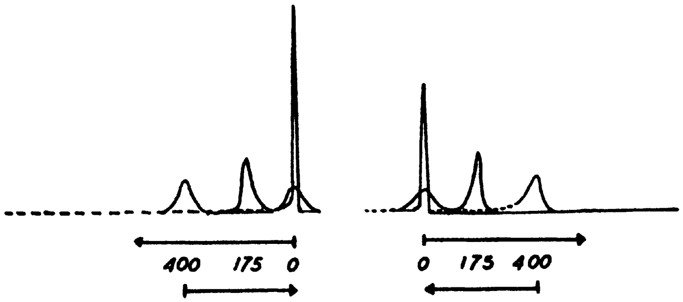

Fig. 4. Electrophoretic boundary spreading at pH 7.6 of *Escherichia coli* bacteriophage T_6 from broth lysate. The patterns are superimposed tracings of Longsworth diagrams; left, descending, right, ascending. The arrows indicate the direction of migration, the numerals, the time in minutes. The upper arrows signify the forward direction, the lower arrows, migration on reversal of the current. The sharp peak at the tail of the arrow is the starting boundary. The dash lines on the protein side of the boundary denote a slightly elevated base line occasioned by light absorption.

interaction of purified thymus desoxyribonucleic acid (DNA) and crystalline serum albumin.[10] Since the areas representing free nucleic acid were unequal on the two sides, the concentrations of this component could not be determined accurately by planimetric integration. In no case did the area exceed 12 per cent on the descending side; in many experiments only traces too small to photograph were observed.

Electrophoretic Purification—To establish the identity of the fast moving component and to provide electrophoretically homogeneous samples of synthetic medium phage for chemical analysis, purification by electrophoretic separation was undertaken. Four experiments with synthetic medium phage concentrated by ethanol precipitation were performed with the 11 ml. separation cell. Electrophoretic diagrams taken just prior to the actual separation will be given in a subsequent paper[1] but are similar to several shown in Fig. 3. On the basis of analytical data[1] as well as from mobility values which more closely coincide with those of desoxyribonucleic acid (DNA), the fast moving component was identified as DNA. In this connection the constancy of the electrophoretic mobility of the fast moving component over the whole pH range covered in Table III is significant. Infectivity measurements and chemical analyses to be reported in a later paper[1] confirmed the assumption that the slow moving component of variable mobility was the bacteriophage.

Electrophoretic Mobility—Since electrophoresis of T_6 bacteriophage could be carried out only above the acid region of precipitation and within the stability zone, it was not possible to determine the isoelectric point by this method. The pH mobility curve for the region pH 5.1 to 8.6 is plotted in Fig. 5. Over the whole range the phage migrated toward the anode. No significant difference in mobility was found between phage from broth and phage from synthetic medium. In a single instance at pH 7.6 the mobility of a preparation from synthetic medium did not fall on the curve given in Fig. 5. Such minor differences may arise from the presence of free nucleic acid in some preparations. Since the mobility of the phage is still -3.55×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$ at pH 5.1, it is difficult to extrapolate to the isoelectric point. However, it seems plausible that this may be in the neighborhood where acid precipitation first takes place, as indicated by the shaded region in Fig. 5. The relatively low mobility of bacteriophage T_6 is significant in view of its extraordinarily high content of nucleic acids, which approaches 40 per cent.[1]

Electron Microscopy—Electron micrographs of purified T_6 bacteriophage prepared from broth cultures by differential centrifugation were kindly made for us by Dr. D. G. Sharp, Duke University School of Medicine. For this work an RCA type B electron microscope was used. A micrograph taken at a magnification of 38,000 is given in Fig. 6. For this experiment the virus dispersed in a phosphate-saline buffer, pH 6.7, was mounted on a collodion film and photographed in the usual manner. The electron micrograph of Fig. 7 was taken by the metallic shadow-casting technique which obscures internal detail but produces sharp contrast. For this experiment 0.25 per cent $CaCl_2$ was added to the virus solution, which was then dried on collodion as usual. The phage particles were then shadowed with chromium by a method previously described (20). The micrographs reveal that there is no gross difference either in size or shape of T_2 and T_6 bacteriophage, both of which consist of tadpole-shaped structures with a slight protrusion at the end of the "tail" and somewhat hexagonal "heads." The particles present a high uniformity of size with the exception of a few which probably represent a phage found as a contaminant only in our early preparations and identified as T_1 on the basis of host range and plaque size.

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[10] Goldwasser, E., and Putnam, F. W., unpublished.

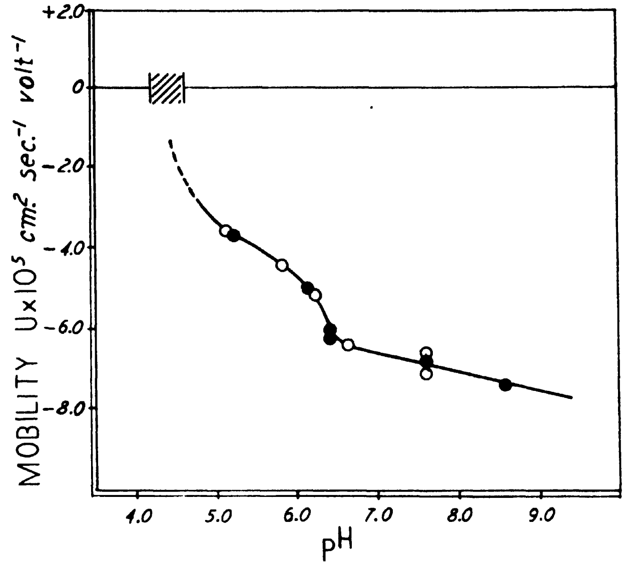

FIG. 5. Electrophoretic mobility curve of *Escherichia coli* bacteriophage T_6 derived from broth (○) and from synthetic medium (●) lysates. The shaded area indicates the inception of the acid region of insolubility and inactivation.

Analysis in Ultracentrifuge—Sedimentation velocity analysis was also performed by Dr. Sharp on the same samples shown in the above electron micrographs. An air-driven analytical ultracentrifuge equipped with the ultraviolet light absorption method was employed. Photographs taken at $2\frac{1}{2}$ minute intervals at a rotor speed of 164 R.P.S. and a mean tempera-

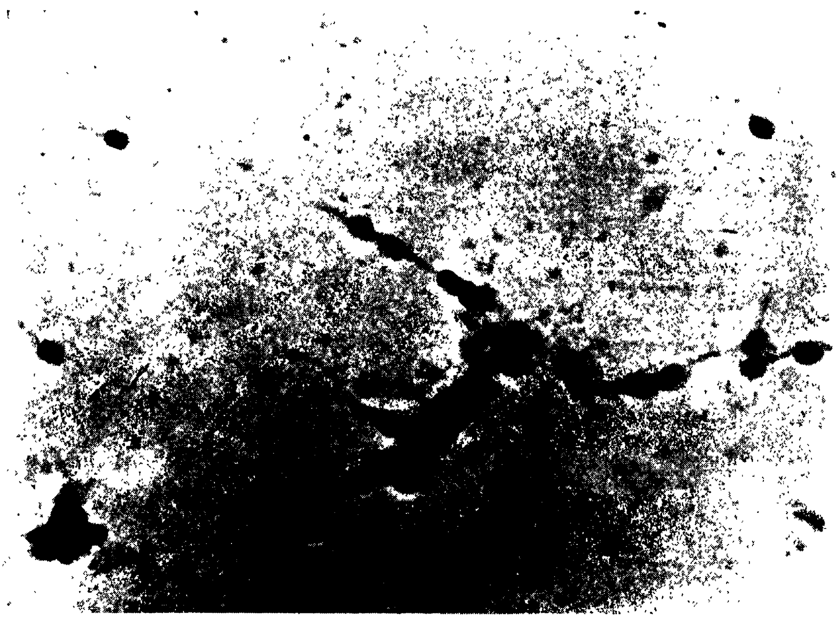

FIG. 6. Electron micrograph of *Escherichia coli* bacteriophage T_6 from broth. The purified phage suspension in phosphate buffer was placed on a collodion film. Magnification 38,000. Photographed by D. G. Sharp.

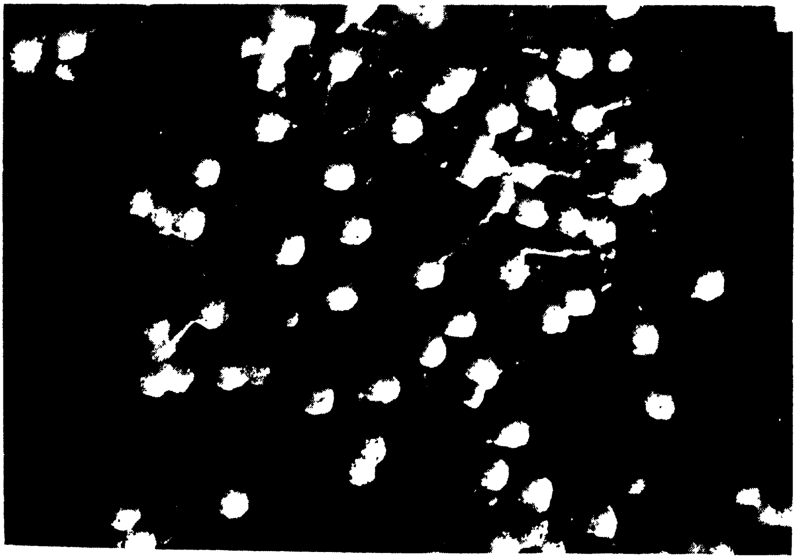

FIG. 7. Electron micrograph of *Escherichia coli* bacteriophage T_6 from broth. The purified phage suspension in 0.25 per cent $CaCl_2$ was prepared as in Fig. 6, and then shadowed with chromium. Magnification 50,000. Photographed by D. G. Sharp.

ture of 25° are given in Fig. 8. Two principal boundaries are visible, and there is indication of a slight almost unsedimentable boundary, presumably nucleic acid. At the time of the analysis, the preparation was more than a month old, and Dr. Sharp has informed us that a similar behavior is shown by old preparations of T_2 bacteriophage. The sedimentation constants for the principal boundaries, corrected to the water basis but neglecting the viscosity of the virus, are 787 and 1034 Svedberg units. Though the values must be considered tentative, they compare closely with the figures reported for the two principal boundaries observed with T_2 bacteriophage, namely 725 and 1030 Svedberg units (8, 9).

An electrically driven analytical ultracentrifuge (Spinco) equipped with a schlieren optical method has recently been made available to us, and we hope to make a sedimentation velocity study of the double boundary phenomenon with T_6 phage in conjunction with electrophoretic studies.

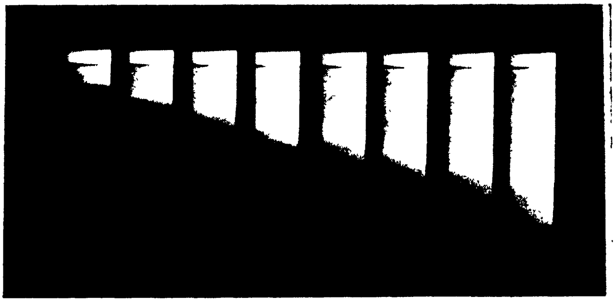

FIG. 8. Sedimentation diagram of *Escherichia coli* bacteriophage T_6 from broth. The concentration of phage was 1.55 mg. per ml. The mean centrifugal acceleration was 7000*g* and the time interval between pictures was 2½ minutes.

In preliminary investigations at pH 7.6 in 0.1 ionic strength veronal-NaCl buffer, a preparation of synthetic medium phage yielded a principal boundary (S_{20} about 700), together with a trace of nucleic acid. Broth phage gave two principal boundaries (S_{20} about 790 and 1010) and a slowly sedimenting boundary, presumably nucleic acid. These values are uncorrected and tentative, but do serve to confirm the order of magnitude of the particle size of bacteriophage T_6.

DISCUSSION

In physical characteristics, in infectivity and stability, and in chemical composition T_6 bacteriophage closely resembles T_2. Moreover, electron micrographs indicate that particles of the two strains of phage are nearly identical in morphology and size. Both phages also exhibit a unique double boundary phenomenon in the ultracentrifuge, the sedimentation constants for the two boundaries for T_6 being the same within experimental error as those for T_2. With T_6 phage, as with T_2, this behavior may be attributed to aggregation, for a small steady increase in phage titer is experienced when high dilutions of the virus in sterile broth are assayed periodically. The average infectivity of T_6 phage, as well as the range of infectivities observed, likewise closely parallels the values observed for T_2. Though the infectivity of various preparations was within rather constant limits, this property may not be considered a sufficient criterion of purity for bacteriophages because of the possibility of aggregation or of the presence of free DNA. From the infectivity value of about 10^{-16} gm. of N per phage and from the N content of 13.2 per cent found by Kjeldahl analysis,[1] it can be calculated that a single virus particle has a mass about 460,000,000 times as great as that of the hydrogen atom.

T_6 bacteriophage derived from synthetic medium cultures differs markedly in stability from T_6 phage harvested from nutrient broth. An increased lability of synthetic medium T_2 phage has likewise been noted by several workers, and has been attributed by Cohen (21) to an external coat of DNA on the synthetic medium phage. Cohen concluded that the apparent external coat was probably not attributable to adsorption of the decomposition product of the phage but rather represented a structural difference; *i.e.*, that the coating was the cause rather than the result of the difference in lability of the virus obtained from the two media. However, the electrophoretic experiments reported herein demonstrate that DNA, which accompanies synthetic medium phage T_6 throughout the course of purification by several methods, is readily separable in an electric field. These data thus suggest that DNA liberated in the decomposition of synthetic medium phage is adsorbed by intact virus particles and is bound by electrostatic forces similar to those involved in the interaction of DNA with simple proteins. The similarity in electrophoretic mobility of T_6 bacteriophage, whether derived from nutrient broth or from synthetic medium, indicates the absence of marked surface structural differences in the two viruses. The adsorption of DNA to the surface of the virus particles is perhaps the factor leading to aggregation and giving rise to the double boundary phenomenon in the ultracentrifuge. The marked effect of Ca^{++} on the sedimentation constant of the virus (8, 9) is in accord with this suggestion.

Electrophoretic analysis served as the chief criterion of physicochemical homogeneity in this investigation. In no instance were more than three components observed. One of these, usually present in trace amounts and more generally associated with synthetic medium phage, was denoted by a fast moving boundary of constant mobility and has been identified as free DNA. In some instances, both with broth phage and synthetic medium phage, two principal boundaries of closely similar mobility were obtained. Preliminary sedimentation velocity analysis suggests that the electrophoresis behavior may be related to the double boundary phenomenon previously reported by Hook *et al.* (8, 9). However, the best preparations of broth phage T_6 yielded a single electrophoretic boundary over the whole pH range of study. Likewise, synthetic medium phage generally exhibited a single slow moving boundary, though always accompanied by small amounts of DNA.

In all electrophoresis experiments, however, the boundary spreading was more rapid than would be expected from free diffusion of particles of the size found for the virus from infectivity data, ultracentrifuge studies, and electron micrographs. The possibility of phage motility was excluded by separate diffusion experiments performed in the Tiselius cell by the Longsworth method (22). While the opalescence of the solutions and boundary skewness precluded precise evaluation of the diffusion constant for bacteriophage T_6, the data revealed that the virus has a low diffusion constant when compared to that of crystalline bovine serum albumin studied simultaneously in the other limb of the cell. In the absence of rapid diffusion, the spreading and skewness of electrophoretic boundaries may generally be attributed to electrophoretic heterogeneity or to thermal convection (23). These effects may be distinguished by reversing the electrical field, boundary sharpness largely being restored in the event that the spreading was due to electrophoretic heterogeneity. However, with broth phage which gave a single skewed boundary at pH 7.6 (Fig. 3, *A*), reversal of the current at reduced power load until the boundaries were restored to their original positions served only to increase their diffuseness (Fig. 4). This result suggests that the diffuseness is not indicative of electrophoretic heterogeneity, but rather of thermal convection occasioned by the low density gradients required by the solubility and optical properties of the virus. However, unusually low power loads were employed in all experiments in order to avert this effect. Accordingly, an alternative hypothesis is suggested: *E. coli* bacteriophage T_6 is apparently the largest particle yet successfully studied in the Tiselius electrophoresis apparatus without the addition of stabilizing proteins. With vaccinal elementary bodies (S_{20} = 4910) boundary streaming was observed which was variously attributed to endosmosis or density gradient effects but could be eliminated by the addition of serum protein (24). Moreover, unlike most known viruses, T_6 coliphage is a tadpole-shaped particle of definite morphology, as revealed by electron micrographs. In addition, there is strong evidence of particle interaction both from sedimentation studies and activity determinations. In view of these facts it seems unlikely that the tailed bacteriophages, even in purified form, would obey rigid physicochemical criteria of homogeneity applicable to small spherical or rod-shaped particles. Both in electrophoresis and in sedimentation the validity of the application of Stokes' law is implied. However, the latter assumes spherical non-interacting particles, a condition obviously abrogated in the bacteriophage system. Likewise, the possibility of preferred orientation due to particle morphology or polarity must be considered, for such orientation would exert a disturbing effect on sedimentation and electrophoresis.

The results of this investigation emphasize the lability and the difficulty of purification of the coliphages and indicate the caution required in subjecting them to physicochemical characterization designed for simple protein molecules. The physicochemical and morphological complexity of the bacteriophages demonstrated herein, together with chemical analyses given in a later paper,[1] is not in accord with the concept that all viruses are simple nucleoproteins. These results, together with those for other viruses summarized in a recent review (25), reveal the inadequacy of the term "molecule" when indiscriminately applied to such infectious agents.

SUMMARY

Escherichia coli bacteriophage T_6 has been grown in quantity after either single or multiple infection of the host maintained in nutrient broth or in synthetic (lactate) medium. The phage was harvested in the supercentrifuge and purified by differential centrifugation, ethanol concentration, or acid precipitation. Bacteriophage prepared from either medium is similar with regard to infectivity, electrophoretic mobility, and pH stability zone, but the synthetic medium phage is markedly more susceptible to inactivation on centrifugation or on standing.

Bacteriophage T_6 prepared by these methods has been characterized by electrophoretic analysis throughout the pH stability zone. Over the region accessible to study the phage migrates toward the anode. Preparations exhibiting a single boundary in the range of pH 5.1 to 7.6 have been obtained from broth cultures by differential centrifugation. However, bacteriophage harvested from synthetic medium has thus far always exhibited at least a trace of a fast moving boundary upon electrophoresis. The impurity has been isolated and identified as desoxyribonucleic acid and is considered to be a decomposition product of the virus which itself contains about 40 per cent DNA. Electron micrographs of bacteriophage T_6 show characteristic tadpole-shaped particles. Preliminary characterization of the purified phage in the analytical ultracentrifuge has revealed the presence of two principal boundaries with sedimentation constants of about 790 and 1030 Svedberg units. As with T_2 bacteriophage, the double boundary phenomenon is attributed to aggregation.

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THE DEPOSITION OF URANIUM IN BONE

IV. ADSORPTION STUDIES IN VITRO*

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Distribution and excretion studies (1–4) have consistently revealed that a large proportion of parenterally administered uranium is deposited in the skeleton from which it is mobilized slowly. It appeared advisable, therefore, to investigate the extent and nature of the affinity of the bone substances for uranium under the more controlled conditions obtainable with preparations *in vitro*.

Bone ash and fresh bone were equilibrated with uranium in a bicarbonate buffer under a variety of conditions, especially those considered to simulate conditions existing in the animal during uranium poisoning. In all cases, a transfer of uranium from the solution to the bone phase was observed. Simultaneously, there was an increase in the amount of calcium dissolved by the buffer. These findings suggested that the mechanism of fixation of uranium involves an ionic exchange with some group (probably calcium) on the surface of the mineral phase. Studies of the solubility of uranium in the solutions employed substantiated the ion exchange hypothesis. No precipitation of uranium (as neutral phosphates) occurred in the range of concentrations of physiological interest.

EXPERIMENTAL

Methods

Bone was taken from the shafts of the long bones of rabbits, freed of soft tissue, and ashed by boiling in a solution of 3 per cent KOH in ethylene glycol (5). After being washed and dried, the ash was ground to a particle size of between 60 and 120 mesh; larger and smaller particles were discarded. Variation in particle size (60 to >200 mesh) was found to have no significant effect on the uptake of uranium; however, particles of intermediate size were the most easily manipulated. Fresh bone was dried, ground to proper particle size, and redried to constant weight.

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* This paper is based on work performed under contract with the United States Atomic Energy Commission at the Atomic Energy Project of The University of Rochester, Rochester, New York.

To simulate conditions *in vivo*, it would have been desirable to employ an ultrafiltrate of blood as the fluid medium. However, because of the difficulty of obtaining adequate quantities of blood ultrafiltrate, bicarbonate buffer at the physiological concentration (0.025 M) was used. With respect to ability to form complex uranyl compounds, bicarbonate is the most important anion in blood ultrafiltrate (6). Though 0.025 M bicarbonate is not an efficient buffer, the pH remained between 7.2 and 7.3 in nearly all cases.

In these studies, all solutions were made by diluting aliquots of a saturated stock solution of uranium containing equimolar quantities of $UO_2(CH_3COO)_2$ and sodium acetate with a concentrated solution of sodium bicarbonate. Sodium acetate was added to reduce the acidity of the uranyl salt; the additional acetate could not introduce a measurable error because of the very low affinity of acetate for uranyl ions (6). The final uranyl bicarbonate solutions were then equilibrated at atmospheric pressure with a mixture of 5 per cent CO_2 and 95 per cent O_2 to adjust the pH. The solution was added to centrifuge tubes containing the bone sample always in the proportion of 1 ml. of solution to 2 mg. of ash and reequilibrated with the CO_2-O_2 mixture. The tubes were then sealed with paraffin-coated corks and shaken at room temperature for 48 hours.

TABLE I

Effect of Buffer on Fixation of Uranium

Medium	Initial solution	Final solution	Uranium removed by bone
	γ U per ml.	*γ U per ml.*	*per cent*
Bicarbonate	24	4.1	83
	24	4.1	83
Serum ultrafiltrate	19	3.9	79
	21	3.9	86

Preliminary tests (7) showed the following. No significant loss of CO_2 occurred during the shaking. There was no adsorption of uranium by the glass or paraffin-coated stoppers. The distribution of uranium between the ash and the solution reached an apparent equilibrium in 48 hours. There was no significant shift in this equilibrium when the equilibration temperature was raised to 37°. There was a marked difference in the uranium uptake if the ratio of weight of bone ash to volume of solution were varied. As indicated in Table I, results obtained with an ultrafiltrate of beef blood as the equilibrating fluid were almost identical with those obtained with the usual bicarbonate buffer.

The solutions, before and after equilibration with bone, were analyzed for uranium by the fluorophotometric method (8) and the differences calculated as the amounts removed by bone. Analyses for calcium by the Roe and Kahn method (9) and phosphorus analyses by the Fiske and Subbarow method (10) were also made on the solutions at equilibrium.

Results

Effect of Varying Uranium Concentration on Transfer of Uranium from Solution to Bone—The essential information on the uptake of uranium by bone ash *in vitro* was obtained by maintaining the bicarbonate concentration at 0.025 M while varying the uranium concentration from 5 to 1500 γ per ml. The distribution of uranium between the mineral phase and the solution at equilibrium as plotted on a log-log scale is shown in the upper curve in Fig. 1. This curve can be divided into three distinct parts (Phases I, II, and III). The concentrations of Ca and P found in these equilibrium solutions are also plotted in Fig. 1, and the curves show three distinct phases coincident with those of the curve of the uranium distribution.

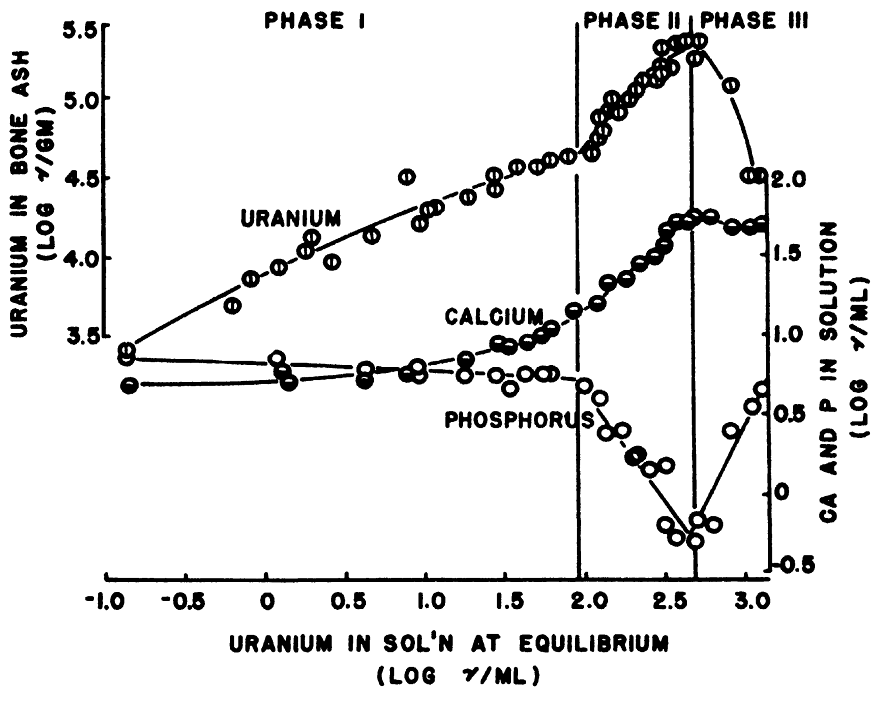

FIG. 1. The adsorption of uranium on bone ash. See the text for explanation.

The disappearance of uranium from solution could be attributed to several factors among which adsorption, precipitation, or ionic exchange seemed most likely. To determine whether the uranium was transferred as a soluble or insoluble compound, a study was made of its solubility in the solutions employed. When uranium was added to solutions of bicarbonate which had been previously saturated with bone ash, precipitates appeared which were found to be a mixture of neutral uranyl phosphates. Accordingly, a more critical study of the solubility of uranyl phosphate in the presence of bicarbonate and calcium was made.

Solubility of Uranium in Saturated Solutions of Bone Ash—The "saturated" solutions were prepared by shaking bone ash for 48 hours with uranium-free, CO_2-equilibrated 0.025 M $NaHCO_3$ in the proportion of 1 ml. of solution to 2 mg. of ash. Such solutions contained consistent quantities of Ca and P, averaging 5.8 and 6.8 γ per ml. respectively. After removing the undissolved ash, uranyl acetate was added to make concentrations varying from 80 to 400 γ of U per ml. The appearance of precipitates in these solutions was erratic but was aided considerably by the addition of solid sodium chloride. Rarely, a flocculent, yellow precipitate appeared immediately in solutions containing as little as 80 γ per ml. of uranium. Occasionally, at uranium concentrations of greater than 200 γ per ml., only a slight turbidity occurred, even after the addition of salt. The precipitates were collected and washed several times with distilled water. Analysis of all precipitates showed that uranium and phosphorus were present in a molecular ratio of approximately 1. A thorough analysis of one such residue showed it to contain 54 per cent uranium, 6.8 per cent phosphorus, 4.3 per cent sodium, 0.88 per cent Ca, and 0.10 per cent magnesium.[1] The amount of carbonate present was probably negligible, since no evolution of carbon dioxide could be detected upon addition of acid. This composition suggested that the precipitate was a mixture of neutral uranyl phosphates such as described by Chretien and Kraft (11). Assuming the presence of 3 moles of water of crystallization per mole of uranium, the composition found agrees with that calculated for a mixture of $Na(UO_2)(PO_4)$, $Ca(UO_2)_2(PO_4)_2$, and $Mg(UO_2)_2(PO_4)_2$ in a molar ratio of 23:2.7:1, respectively. Since the precipitates were formed by a drastic, salting-out procedure, it cannot be claimed that precipitates formed on the bone surface would have comparable proportions of the cations. However, the results demonstrate that the formation of such mixtures of uranyl phosphates in the equilibrium solutions is possible. Such precipitates, if formed in the experiments described above, would increase the quantity of uranium calculated as that removed by bone.

Solubility of Uranium in Synthetic Bone Ash Solutions—The solubility behavior of uranyl phosphates was investigated quantitatively in synthetic solutions in which Ca and P were maintained at the concentrations found in the saturated solutions of bone ash; *i.e.*, bicarbonate (as $NaHCO_3$) at 0.025 M and calcium (as $CaCl_2$) and phosphorus (as Na_2HPO_4) each at 6 γ per ml. The range of concentrations of uranium (as $UO_2(CH_3COO)_2$) employed was the same as in the previous bone ash experiments, *i.e.* from 27 to 1500 γ of U per ml. The solutions (whether or not precipitates were present) were equilibrated with 5 per cent CO_2-95 per cent O_2 at atmospheric pressure and allowed to stand for 3 days. They were then centrifuged without the addition of salt and the supernatant solutions were analyzed for uranium and phosphorus. These data are, in part, presented in Fig. 2.

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[1] Mg was determined spectrographically by Dr. L. Steadman. Na was determined by Dr. J. Holler using a flame photometer.

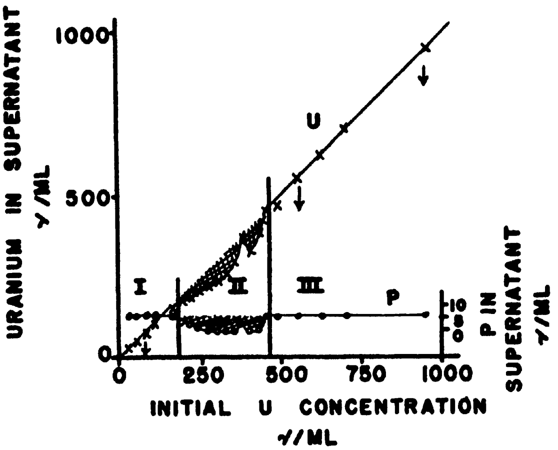

FIG. 2. The precipitation of uranyl phosphate from bicarbonate buffer at various concentrations of uranium.

As in the case of the bone ash-uranyl bicarbonate system, and at equivalent concentrations of uranium, the solubility results may be divided into three phases (Fig. 2). In solutions containing uranium at concentrations of 150 to 450 γ per ml., precipitates of uranyl phosphates exhibiting U:P ratios of approximately 1 appeared consistently as indicated by the cross-hatched areas. In a few tests, precipitation occurred outside of these concentration limits (indicated by arrows). If, however, sodium chloride were added, precipitation took place at all uranium concentrations above 80 γ per ml. Apparently, between 80 and 150 γ per ml. and above 450 γ per ml., the precipitate tended to remain suspended in a colloidal state.

This information clarified the results presented in Fig. 1. During Phase I, below 80 γ of U per ml., uranium remained soluble. It was, however, taken up by the ash, possibly in exchange for Ca^{++}, since the amount of calcium in the solution increased as more uranium was taken up by the ash phase. During Phase II, the solubility of uranium was exceeded and uranyl phosphates precipitated on the bone particles, resulting in a sharp, simultaneous loss of uranium and phosphate from solution, with an increase in calcium from further solution of bone as phosphate was removed from solution. During Phase III, the precipitation was reversed, and greater quantities of uranium and phosphate were found in the solutions at equilibrium, probably as colloidal uranyl phosphates, since calcium remained elevated.

Reversibility of in Vitro System—The reversibility of the bone ash-bicarbonate buffer system was demonstrated in the following experiment. After the usual 48 hour equilibration with uranium, the supernatant solution was aspirated from the bone and replaced by an equivalent amount of bicarbonate solution which was uranium-free but had previously been equilibrated with bone ash. The tubes were then shaken for another 48 hours by which time a new equilibrium was shown to have been attained.

TABLE II

Reversibility of Bone Ash-Bicarbonate Buffer System

Total uranium present initially in bone	Uranium in solution at equilibrium	Uranium in bone at equilibrium	Theoretical for bone at 2nd equilibrium (from Fig. 1)
*γ per ml.**	*γ per ml.*	*mg. per gm. ash*	*mg. per gm. ash*
21.4	1.1	10.2	10.0
99	24	37.5	34.0

* The concentration of uranium initially present in bone was calculated as its equivalent in γ per ml. of solution for purposes of comparison.

The uranium concentrations in the solution initially added to impregnate the bone and in the two successive equilibrium solutions were determined. With these data, calculations could be made of the amount of uranium which was taken up and the amount subsequently given off by the bone ash. If the equilibrium were reversible, the final bone to solution distribution of uranium would be the same in systems containing an equivalent amount of uranium whether that uranium resided initially in the bone or in the solution. Data obtained from the curve in Fig. 1 served as a comparison and are given in the last column of Table II. Within the limits of experimental error, the results obtained from two experiments indicated a complete reversibility.

Comparison of Uranium Fixation in Fresh and Ashed Bone—In a few experiments, fresh bone was substituted for bone ash. Such an experiment is reported in Table III. In view of the fact that ash constitutes 70 per cent of the fresh, dried bone, it was surprising that fresh bone removed only 12 per cent as much uranium as did bone ash. It was found that a 7-fold greater quantity of fresh bone (14 mg.) was approximately equivalent in adsorptive power to the usual amount (2 mg.) of bone ash.

Because of the lower affinity of fresh bone for uranium, there seemed reasonable doubt that the ion transfer mechanism observed *in vitro* could account for the skeletal deposition of uranium *in vivo*. In rats, following an injection of 2.5 mg. of U per kilo, the blood uranium decreases from an estimated 36 γ of U per ml. to less than 0.1 γ of U per ml. in a few hours, in which time 60 per cent of the dose appears in the urine (1). Simultaneously, the remaining 40 per cent is deposited principally in the skeleton, resulting in concentrations as high as 50 γ of U per gm. of bone ash (1). To duplicate these conditions roughly, the following experiment was conducted. Assuming the rat to consist of 25 per cent extracellular fluid and 6 per cent bone (dry weight), the ratio of bone to fluid is roughly 1:4. Powdered, fresh bone was added in a ratio of 1:4 to bicarbonate buffer containing 14 γ of U per ml. (40 per cent of 36 γ per ml.). At equilibrium, the concentrations in the bone and solution were 56 γ of U per gm. of ash and 0.053 γ of U per ml. respectively, duplicating the results of studies *in vivo*.

TABLE III

Comparison of Fresh and Ashed Bone with Respect to Fixation of Uranium

Uranium in solution initially, 38 γ per ml.

	No. of samples	Uranium in bone at equilibrium
		mg. per gm.
Fresh bone	12	3.4
Ashed "	From curve in Fig. 1	13.5

DISCUSSION

Over the range of concentrations observed in blood during uranium poisoning (1) (corresponding to Phase I) uranium was found to be completely soluble under our experimental conditions. None the less, uranium was transferred from solution to bone. The distribution of uranium between solution and bone was found to be dependent upon the relative amounts of the two phases, suggesting a surface-limited reaction. The fact that the equilibrium was relatively unaffected by variations in particle size might be considered as contrary evidence were it not for the demonstration (12) that the surface area of bone ash is not a function of particle size. As seen in Fig. 3, the data, plotted as a typical sigmoid curve (interrupted by Phase II), suggested that the equilibrium, at these concentrations, conformed to the laws governing dissociation. Further, the transfer was found to be completely reversible, and was insignificantly affected by temperature changes.

A surface-limited reaction involving dissociation is suggestive of an ion exchange process. Such a process has been demonstrated for the interaction between bone ash and phosphate buffer (12).

If an ion exchange is responsible for the transfer of uranium from solution to bone, the ion most likely to be exchanged for UO_2^{++} is Ca^{++}. That calcium took such a part in the exchange was suggested by the increased calcium content of the equilibrium solutions when greater quantities of uranium were added to the bone ash-buffer system. Subsequent studies have confirmed this suggestion.

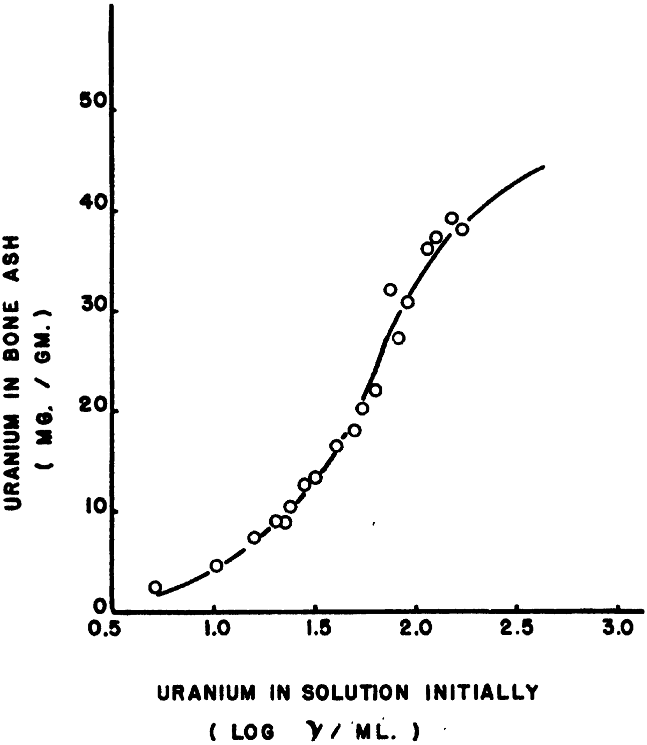

FIG. 3. The relation between concentration of uranium in solution and in bone ash plotted as a dissociation phenomenon.

The maximum quantity of uranium held by bone ash may be estimated from Fig. 3 to be of the order of 48 mg. per gm. or 0.2 mM per gm. The only anionic grouping present in the bone surface in a concentration high enough to account for this binding capacity is phosphate, 0.55 mM per gm. (13).

The equilibrium distribution of uranium between bone and solution was strongly in favor of bone. At the lowest point on the curve (Fig. 1) where there was insufficient uranium to satisfy the available binding capacity of bone, the concentration of uranium in ash was 20,000 times that in solution. Even as the binding capacity approached saturation (end of Phase I), the distribution favored bone by a factor of 400. Thus, the ionic transfer of uranium from solution to bone is of such a magnitude as to account quantitatively for the deposition of uranium in the skeleton *in vivo.*

SUMMARY

Bone ash and fresh bone were equilibrated with uranium in a bicarbonate buffer under a variety of conditions, especially those considered to simulate conditions existing in the animal during uranium poisoning. Observations of the transfer of uranium from the solution to the bone phase and the simultaneous solution of calcium and phosphorus suggested that the mechanism of fixation at concentrations of uranium occurring *in vivo* involves an ionic exchange between uranium in solution and some group (probably Ca) on the surface of the bone crystals. This was substantiated by a study of the solubility of uranium in the solutions employed. The ionic exchange occurring *in vitro* quantitatively accounts for the deposition of uranium in the animal skeleton.

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THE DEPOSITION OF URANIUM IN BONE

V. ION EXCHANGE STUDIES*

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Studies of the transfer of uranium from bicarbonate buffer to various bone preparations *in vitro* (1) indicated that the fixation of uranium was for the most part a surface phenomenon involving ionic exchange. It was also noted that two different bone preparations varied markedly in their ability to adsorb uranium. Glycol-ashed bone was much more effective than fresh bone of the same particle size.

This is a report of a series of investigations of the ionic exchange mechanism. First, a comparison was made of the ability of three bone preparations to adsorb uranium *in vitro* with their ability to exchange phosphate and calcium ions. Second, a study was made of the effect of a preliminary saturation of glycol ash with uranium on its phosphate and calcium exchangeability. The results from both investigations confirmed the view that uranium fixation by bone does involve an ionic exchange.

EXPERIMENTAL

Methods

Three bone preparations from the shafts of the long bones of rabbits were tested: (*a*) fresh bone, dried to constant weight, and ground to a particle size between 60 and 120 mesh; (*b*) glycol ash, obtained by boiling fresh bone in alkaline glycol at a temperature of approximately 200° (2), also ground to a 60 to 120 mesh size; (*c*) "glycol-muffle ash," obtained by heating glycol ash in a muffle furnace at 400° for 6 hours followed by 6 hours at 700°.

The method of equilibrating bone preparations with bicarbonate buffer containing uranium was the same as that reported earlier (1). Phosphate exchange was studied with the techniques reported by Falkenheim, Neuman, and Hodge (3) in which an equilibration of the bone preparation at pH 7.3 with 0.0013 M phosphate buffer containing P^{32} is used. Similar

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* This paper is based on work performed under contract with the United States Atomic Energy Commission at the Atomic Energy Project of The University of Rochester, Rochester, New York.

procedures[1] were used for calcium exchange except that the equilibration solution was unbuffered (pH 6.7) and contained only calcium chloride with added Ca^{45}.

A modification[2] in the method of calculating exchange data (3) was made. This modification corrects for small order changes in the concentration of the exchangeable ion in the solution phase which occur occasionally during equilibration.

Uranium was determined by the fluorophotometric method (4), phosphate by the method of Fiske and Subbarow (5), and calcium by the method of Roe and Kahn (6).

Results

It was noted that bone preparations which had been ashed in alkaline glycol removed uranium from bicarbonate buffer, whereas bone preparations ashed at high temperature in a muffle furnace did not. To clarify this problem, a glycol ash preparation was further treated in a muffle furnace at 700° and the uranium uptake and phosphate and calcium exchange of this preparation were compared with that of the original glycol ash. The results are given in Table I. Within the limits of experimental

TABLE I

Effect of High Temperature Ashing on Uptake of Uranium and Exchange of Phosphorus and Calcium

Preparation	U uptake*	P exchange†	Ca exchange†
	mg. per gm. ash	*per cent*	*per cent*
Glycol ash	37	8.8	13.8
Glycol-muffle ash	0	0.5	2.3

Each value represents an average of three samples.

* Initial concentration in solution was 100 γ per ml.

† The exchange values were obtained from a 48 hour equilibration.

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[1] Falkenheim, M., Underwood, E., and Hodge, H. C., unpublished results.

[2] The earlier (3) method of calculation was based on the assumption, that, at equilibrium, the ratio of radioactive to normal isotope was identical in the two systems: in the solution and in the exchangeable fraction of the insoluble phase. The new calculation is based on the standard "isotope dilution" technique. The assumption in this case is that, at equilibrium, the dilution of the radioisotope to the normal isotope in the solution is a measure of the amount of normal isotope contributed by the insoluble phase. The calculation is $C_B = ((C_e \times R_i)/R_e) - C_i$, where C_B = mg. of exchangeable ion in the bone phase, C_e = mg. of exchangeable ion in the solution phase at equilibrium, R_i = total radioactivity of ion added to solution phase initially, R_e = total radioactivity remaining in the solution phase at equilibrium, and C_i = mg. of exchangeable ion in the solution phase initially. From the equation above, the per cent ion in bone which is exchangeable = (C_B/(mg. total ion in the bone phase)) × 100.

error, the high temperature treatment completely prevented the transfer of uranium from the buffer to bone. Calcium exchange was markedly reduced and phosphate exchange nearly abolished.

As noted previously (1), fresh bone was less effective than glycol-ashed bone in binding uranium *in vitro*. The phosphate and calcium exchange of this fresh bone was also studied. To insure that the lower results reported previously could not be attributed to a slower rate of reaction, the adsorption of uranium and the exchange of calcium and phosphate were studied over a period of 6 to 8 days. The data are presented graphically in Fig. 1. Although not reduced as markedly as the uranium uptake, both calcium and phosphate exchanges were much lower in the fresh bone preparation.

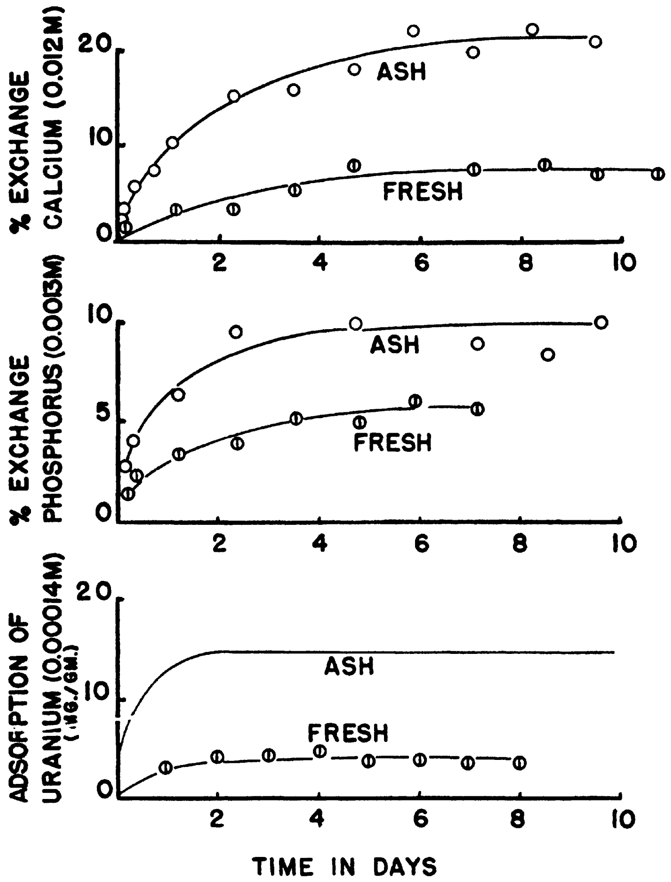

FIG. 1. A comparison of the ability of ashed and fresh bone to adsorb uranium and exchange calcium and phosphate. The curve for uranium uptake by ash is taken from previous studies (1).

If uranium were adsorbed on the bone surface by a mechanism involving ionic exchange, it would follow that the ion for which the uranium exchanged would be reduced in concentration in uranium-treated bone. To determine the effect of uranium impregnation on calcium and phosphate exchange, two samples of glycol-ashed bone were first equilibrated with uranium in bicarbonate buffer for 48 hours, the solution aspirated, and the impregnated samples then studied for calcium and phosphate exchange. The uranium content of the bone used for calcium exchange was calculated to be 31 mg. of U per gm. and that of the bone used for phosphate exchange, 26 mg. of U per gm.

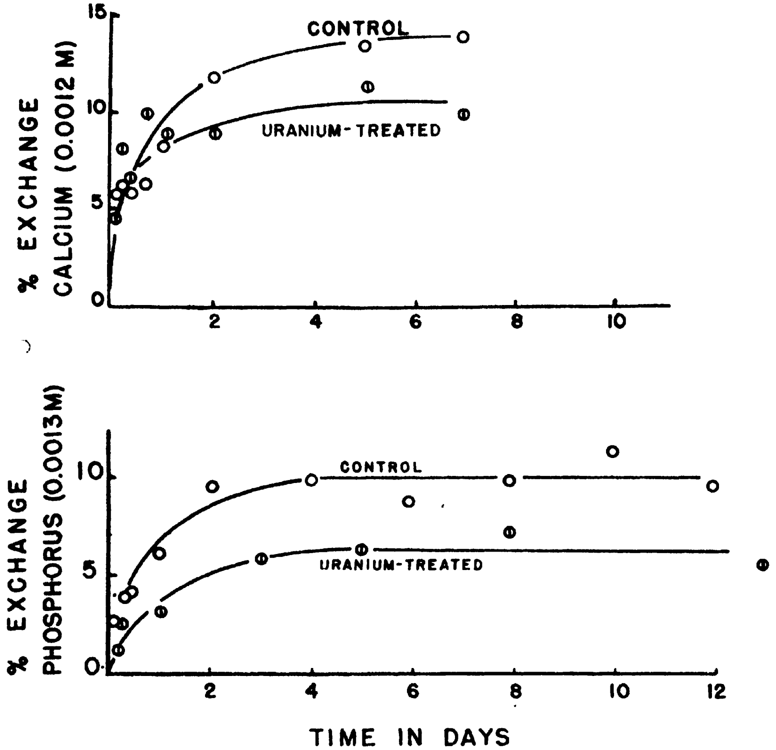

FIG. 2. The effect of uranium impregnation on the ability of bone ash to exchange calcium and phosphate.

The presence of uranium inhibited the exchange of *both* calcium and phosphate, as seen in Fig. 2. The adsorption of 1 mole of uranium reduced the amount of exchangeable phosphate *and* the exchangeable calcium each by 2 moles.[3]

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[3] This calculation is subject to rather large error. The observed values were P, 1.97 ± 0.4; Ca, 2.1 ± 0.3.

DISCUSSION

The observation that high temperature ashing prevents the fixation of uranium by bone confirms the results of previous solubility studies (1) which showed that the fixation process did not involve a precipitation of uranyl phosphate. The change in composition of the insoluble phase (bone ash) resulting from high temperature treatment would not be expected to influence a precipitation reaction so markedly.

On the other hand, all of the experiments described in this report demonstrate the correlation between the amounts of surface calcium and phosphate (exchangeable) in the various bone preparations and their ability to fix uranium. This strengthens the view that the mechanism of fixation involves ionic exchange.

The ion in the bone surface most likely to be exchangeable with the uranyl ion (UO_2^{++}) would be calcium (1). It would be expected, therefore, that bone samples impregnated with uranium would contain a smaller amount of exchangeable calcium. As indicated in Fig. 2, this was found to be true. It was surprising, however, that 1 mole of uranium replaced 2 moles of calcium. Both are divalent ions. It was also surprising that surface phosphate, too, was rendered non-exchangeable to the extent of 2 moles per mole of uranium bound by the bone. The significance of this finding will be discussed in a later publication (7).

Apart from the problem of uranium deposition, data have been obtained bearing on the crystalline structure of bone mineral. On a percentage basis, approximately twice as much calcium as phosphate was found to be exchangeable, indicating that the surface of the crystal contains a higher concentration of calcium than does the interior. A simple calculation shows the Ca:P ratio in the surface is of the order of 4. Most of the non-phosphate anions in bone must therefore be associated with surface calcium, an idea expressed 8 years ago (8). Further studies on this interesting problem are desirable.

SUMMARY

Previous studies have indicated that the fixation of uranium by bone is, for the most part, a surface phenomenon involving ionic exchange. The results in this report confirm this view. First, a correlation was demonstrated between the magnitude of the fraction of exchangeable calcium and phosphate of bone preparations and their ability to fix uranium. Second, the presence of adsorbed uranium inhibited the exchange of calcium and phosphate of bone.

The authors gratefully acknowledge the assistance of E. Underwood in the analysis of calcium and radioactivity measurements. She kindly furnished the data on calcium exchange presented in Fig. 1.

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THE DEPOSITION OF URANIUM IN BONE

VI. ION COMPETITION STUDIES*

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Previous studies have indicated that the deposition of uranium in bone is a surface-limited reaction involving an exchange of the uranyl ions of the solution for some ion in the surface of the mineral phase of bone, probably calcium (1, 2). As a further test of the exchange hypothesis, a series of ion competition experiments was conducted, in which the effects of varying concentrations of hydrogen, bicarbonate, phosphate, and calcium ions were observed. Evidence was obtained which confirmed the exchange hypothesis and established calcium as the principal surface ion in bone which uranium displaces.

EXPERIMENTAL

Methods

In these experiments, only bone which had been ashed in alkaline glycol (3) at 200° was employed. The method of equilibrating bone ash with bicarbonate buffer containing uranium has been reported earlier (1). Analyses for uranium were made by the fluorophotometric method (4), phosphorus by the method of Fiske and Subbarow (5), and calcium by the Roe and Kahn (6) procedure.

Results

Uranium Deposition in Bone Ash with Varying Concentrations of Bicarbonate and Hydrogen Ions—When the concentration of uranium was kept constant (at 87 γ per ml.) and the concentration of bicarbonate increased in the solution phase, decreasing quantities of uranium were taken up by the bone ash. A semilog plot of these data including the pH changes involved is presented in Fig. 1.

The decrease in uranium uptake was due, in part, to the increase in pH coincident with increasing concentrations of bicarbonate. This was

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* This paper is based on work performed under contract with the United States Atomic Energy Commission at the Atomic Energy Project of The University of Rochester, Rochester, New York.

demonstrated by lowering the pH of a *concentrated* bicarbonate medium to 6.7 by equilibration with 100 per cent CO_2. The uranium deposition was

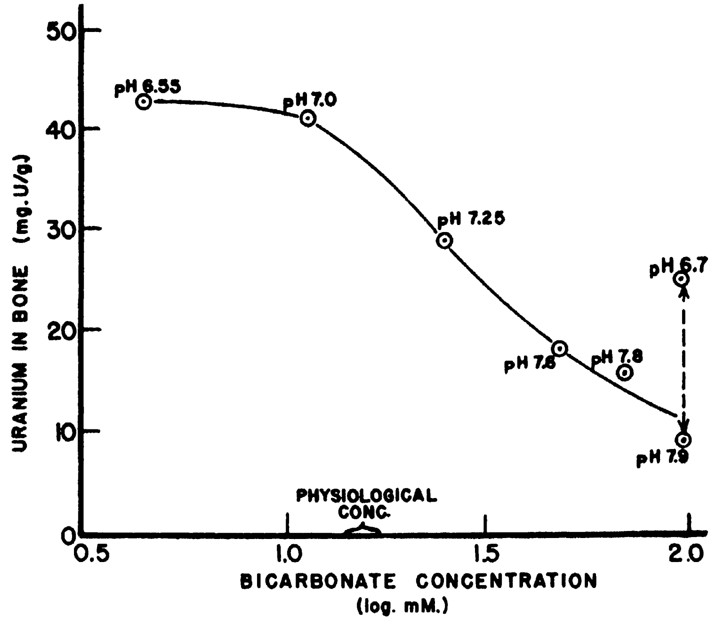

FIG. 1. The effect of bicarbonate concentration on uranium deposition

TABLE I

Effect of pH on Uranium Uptake by Bone Ash

Initial concentration of uranium in solution	pH	Concentration of uranium in bone*
γ per ml.		*mg. per gm.*
45	7.45	17
	8.00	12.7
114	7.51	35
	8.00	26
145	6.00	71
	7.30	44.5

* Each value represents an average of two samples.

then shifted toward the theoretical level for a system containing ***dilute*** bicarbonate at pH 6.7 (equilibration with the usual 5 per cent CO_2).

This pH effect was confirmed in preparations of 0.025 M bicarbonate containing various concentrations of CO_2 and uranium. Equilibration with different concentrations of CO_2 would not materially alter the bi-

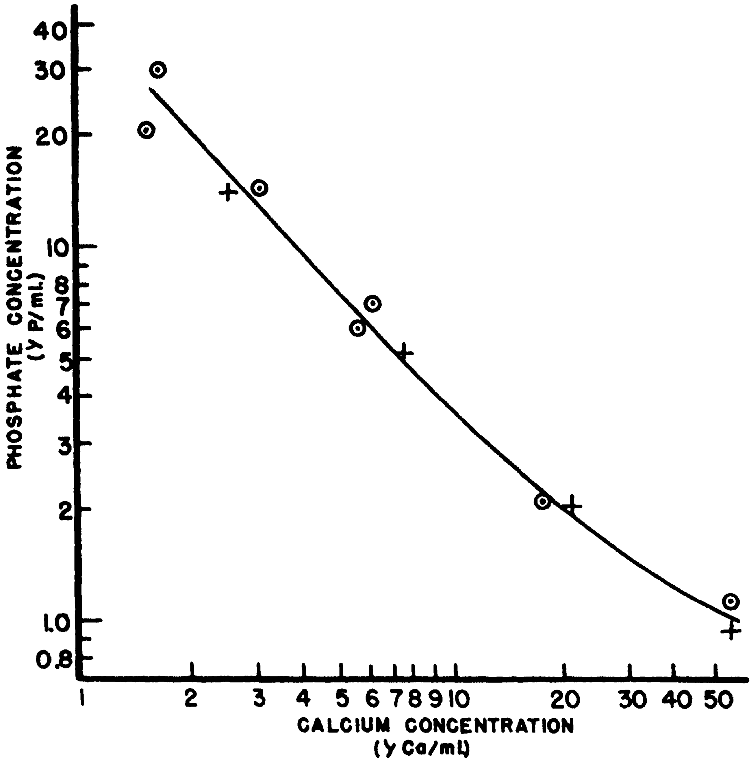

FIG. 2. The relationship between calcium and phosphate concentrations in the equilibrium solution. O designates values obtained in uranium-free bicarbonate buffer; +, values obtained in bicarbonate buffer containing 3 to 18 γ of U per ml. at equilibrium.

TABLE II

Displacement of Bone Calcium by Uranium

Initial concentration of uranium in solution	Concentration of Ca in solution at equilibrium	Concentration of PO_4 in solution at equilibrium
γ per ml.	*γ per ml.*	*γ per ml.*
0*	0.74	6.5
	(0.42–1.2)	(6.2–7.0)
46	5.1	6.5
114	6.5	6.0
145	7.3	7.0

* Average of six values; all others an average of two samples.

carbonate concentration as it changed the pH. The results are presented in Table I.

Uranium Deposition in Bone Ash with Varying Concentrations of Calcium *in Solution*—To a series of solutions in which the uranium (45 γ per ml.) and the bicarbonate concentrations (0.025 M) were kept constant, varying quantities of $CaCl_2$ or Na_2HPO_4 were added. Excess calcium ions reduced the amount of uranium taken up by bone and also reduced the phosphorus in solution. At equilibrium, as illustrated in Fig. 2, an inverse relationship (log-log plot) was observed between the concentration of calcium and phosphate. Therefore, although an increase in the Ca:P ratio markedly decreased the deposition of uranium, it was impossible to demonstrate whether the effect could be attributed to calcium or phosphate. It was necessary to examine correlative data.

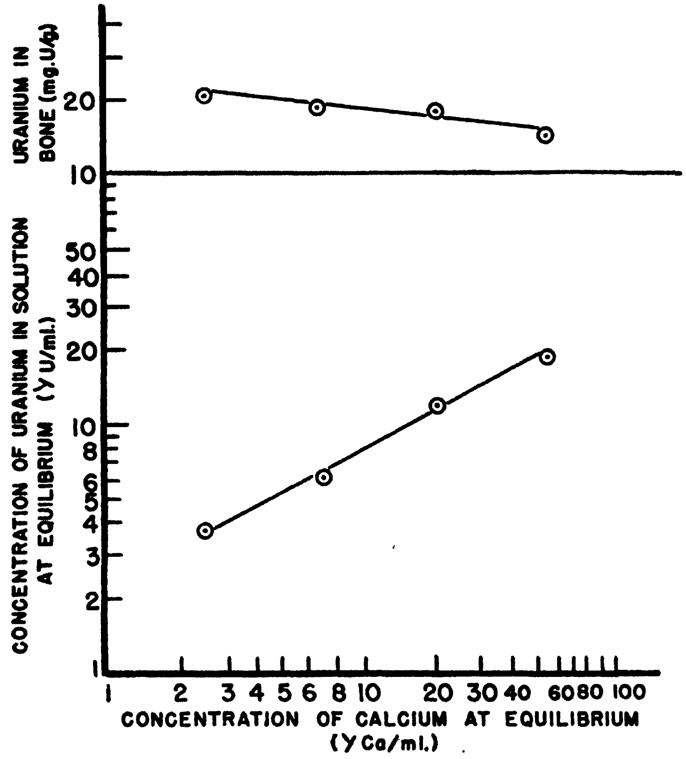

FIG. 3. Data illustrating the direct competition between calcium and uranyl ions for bone.

It was observed that, at pH 8, the progressive addition of uranium to the system invariably resulted in an increase in the concentration of calcium in the equilibrium solution without the usual corresponding decrease in the phosphate concentration. These data are assembled in Table II.

Therefore, it seems reasonable to assume that, at pH 7.3, the effect of varying Ca:P ratios on the deposition of uranium may be due to variations in the calcium ion. The concurrent changes in phosphate concentration may be attributed to the limited solubility of some compound of calcium and phosphate.

With this assumption, it is evident that a direct competition exists between calcium and uranium for the combining sites on the bone phase. Fig. 3 illustrates this competition by showing the linear relationship (log-log plot) between the concentrations of calcium and uranium in the equilibrium solution and the inverse relationship between calcium in solution and uranium in bone.

DISCUSSION

With the observations reported above, sufficient data (1, 2, 7, 8) have now been accumulated to permit a general description of the mechanism by which uranium is deposited in bone.

The mechanism of uranium's nephrotoxic effects has been recently reviewed (9). A brief summary of this information is pertinent to the present discussion. To elucidate the mechanism of the toxicological action of uranium, Dounce and Flagg (10) investigated the chemical behavior of uranium under physiological conditions. Their studies demonstrated that very little uranyl ion (UO_2^{++}) exists in solution at pH 7.2. Rather, most of the uranium forms complexes with various organic and inorganic anions. The complexes of uranium with proteinate, bicarbonate, and acetate were investigated in detail; only preliminary studies were made of complexes of uranium with malate, citrate, and organic and inorganic phosphates. Based on these chemical studies, the metabolism of hexavalent uranium was formulated as follows: In the animal, uranium is carried in the blood principally in two forms, an indiffusible fraction which is protein-bound (about 40 per cent) and a diffusible fraction consisting largely of the uranyl bicarbonate complex (about 60 per cent). These two fractions are in equilibrium, so that removal of the diffusible portion results in a dissociation of the protein-bound fraction. Under physiological conditions, the uranium in combining with protein causes little or no denaturation. Cellular damage occurs *in vivo* only when there is insufficient bicarbonate and other organic anions to keep uranium in the form of a diffusible complex ion, *viz.* in the kidney tubule. The diffusible bicarbonate complex of uranium is filtered through the glomerulus and as the glomerular filtrate passes through the tubule, bicarbonate ion is resorbed. Concurrent with the fall in bicarbonate concentration, the dissociated uranyl ion is free to combine with active groups on the surfaces of the cells lining the tubules, ultimately resulting in cellular damage.

Data on the distribution and excretion of administered uranium (11) showed that uranium is deposited *in vivo* in two principal sites: the kidney and the skeleton. The mechanism of the deposition of uranium in kidney has been adequately explained (9), as reviewed above. It was the purpose of our studies to elucidate the mechanism by which administered uranium becomes fixed in the skeletal system.

It has not been established whether cellular activity takes part in the fixation of uranium by bone *in vivo*. However, the equilibration of ground bone (fresh and ashed) with dilute solutions of uranium *in vitro* has consistently resulted in a transfer of uranium from the solution to the solid phase. Further, the order of magnitude of this transfer was such that any process involving cellular activity *per se* must be considered quantitatively unimportant (1).

Studies of the solubilities of uranyl phosphates (1) have revealed that, in the presence of bicarbonate, uranyl phosphates precipitate only at concentrations of uranium higher than those attained *in vivo*.

Thus, some form of surface activity appeared responsible for the fixation process. Substantiating evidence was required, together with the identification of the surface group or groups involved.

It was found that the distribution of uranium between the solution and the solid phase was a function of the ratio of the amounts of the two phases (1), strongly indicating a phase-limited reaction. When the ratio of solution to bone ash was kept constant, a plot of the amount of uranium added against the amount of uranium adsorbed by bone resulted in an S-shaped curve, indicating that the phenomenon followed the law of mass action. The dissociation of the bone-uranium combination, however, was extremely low. Confirming the dissociation concept was the observation that the equilibrium was reversible and relatively unaffected by temperature variation (1).

The principal surface groups capable of chemical union with uranium can be listed: protein carboxyl, carbonate, and phosphate. Protein carboxyl can be eliminated for three reasons: (*a*) the number of groups could account for only a few per cent of the total binding capacity; (*b*) ashed bone, free of protein, was more efficient in adsorbing uranium than fresh bone (1); and (*c*) protein-bound uranium has been found to be highly dissociated (10). Carbonate groups could not be responsible for the affinity of the bone mineral for uranium for two reasons: (*a*) the number of carbonate groups present in bone could account for only a few per cent of the total binding capacity, and (*b*) since uranyl complexes of the type $UO_2—O—CO—R$ are highly dissociated (10), it may be concluded that carbonate groups of the bone surface can bind uranium only by forming a complex of a more complicated multivalent type. In solutions containing an excess of either bicarbonate or carbonate ions, uranium forms a complex ion of the type $UO_2(CO_3)_2^{=}$ or possible $UO_2(CO_3)_3^{\equiv}$ (10), which are relatively undissociated. Though such a complex ion might be physically adsorbed on a mineral surface, it seems unlikely from spatial considerations that such a complex could be formed with separated carbonate groupings in a highly oriented crystal lattice.

The evidence favored the implication of surface phosphate groups. Further indication of this was the demonstration of a good correlation between the number of surface (exchangeable) phosphate groups in various bone preparations and their respective affinities for uranium (2). Orthophosphate forms a relatively dissociated[1] though sparingly soluble complex with uranium. At first consideration, this finding appears incompatible with the low dissociation of the bone-uranium combination (1). However, uranyl pyrophosphate has been demonstrated[1] to be only slightly dissociated (K about 5×10^{-8}). Since it has been found that the fixation of 1 mole of uranium renders 2 moles of the bone's surface phosphate nonexchangeable (2), it appears likely that uranium combines with two *adjacent* phosphate groups in the mineral surface, structurally analogous to uranyl pyrophosphate. Existing data (11) on the arrangement of phosphate groups in the apatite crystal indicate that such a combination is structurally feasible. Quantitative considerations support this hypothesis. Recalculation of data presented earlier (1) shows that the maximum capacity of bone ash for uranium is approximately 40 per cent of the number of surface (exchangeable) phosphate groups. This is in surprising agreement with the theoretical limit of 50 per cent, if one considers the possible space limitations.

In combining with surface phosphate groups, uranium must displace some cation or cations. The weight of evidence (1, 2) indicated that calcium was the principal cation undergoing exchange with uranyl ion. The demonstration of a direct competition between calcium and uranium for the bone surface substantiates this view.

As in the case of calcium, increasing the concentration of bicarbonate in solution reduced the amount of uranium taken up by bone ash. This can be interpreted as direct competition between bicarbonate ion (which forms a relatively undissociated complex with uranyl ion (10)) and the surface phosphate group for uranium. The reduced uranium adsorption observed at elevated pH is somewhat more complicated but, in part, may be viewed as a competition between surface phosphate groups and hydroxyl ions. These results, though interesting from a theoretical standpoint, have proved of little practical significance. Alkali feeding did not increase the rate of removal of uranium deposited in the skeleton of the rat (7). High calcium diets have not, as yet, been tested.

The mechanism described above for the fixation of uranium by bone is based entirely upon the results of studies of bone preparations *in vitro*. In translating this mechanism to the situation obtaining *in vivo*, many additional variables are encountered. Such factors as crystal size, crystal growth, recrystallization rate, rate of fluid exchange, etc., may be expected to vary from one area to another. These variables may be expected to alter quantitative aspects of the deposition resulting in an unequal distribution of uranium throughout the skeleton similar to that actually observed (8).

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[1] W. F. Neuman and J. Havill, unpublished polarographic studies; also, A. Rothstein and R. Meier, unpublished results.

SUMMARY

In studying the deposition of uranium in bone, a series of ion competition experiments was conducted, in which the effects of varying concentrations of hydrogen, bicarbonate, phosphate, and calcium ions were observed. Evidence was obtained which confirmed the hypothesis that calcium is the surface ion in bone which uranium displaces. All available data have been summarized and the mechanism of the deposition of uranium in bone is discussed in detail.

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STUDIES OF THE COMPOSITION OF THE LIVETIN FRACTION OF THE YOLK OF HEN'S EGGS WITH THE USE OF ELECTROPHORETIC ANALYSIS

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Investigations of the antigens of rickettsiae grown in the yolk sacs of embryonated hen's eggs have led us to the examination of the yolk of non-embryonated eggs (1, 2). The proteins present in the yolk are generally considered as two fractions, lipovitellin and livetin, which are differentiated according to their solubility in salt solutions. When an egg yolk suspension containing 10 per cent sodium chloride is extracted with ethyl ether, a clear aqueous solution results. If the solution is dialyzed against distilled water, or poured into 9 volumes of distilled water, a precipitate forms which has been called lipovitellin. This lipoprotein has been studied, for example, by Chargaff (3) who was able to obtain preparations of constant composition. The Sharples supercentrifuge has been employed by Alderton and Fevold (4) to precipitate lipovitellin from egg yolk, and the effluent has been shown by Fevold and Lausten (5) to contain another lipoprotein, termed lipovitellenin, which is soluble only in 10 per cent sodium chloride saturated with ether. Few reports, however, could be found on that portion of egg yolk which is soluble at salt concentrations near isotonicity, and it is this material which one encounters in many antigens prepared from infected yolk sacs.

In the present work on the livetin fraction, electrophoretic analysis has been frequently applied and, because clear solutions were essential, considerable time was spent in developing methods of preparation. Although it was possible to obtain sufficiently clear preparations by adequate ether extraction, an additional method was developed whereby clear solutions were prepared without ether extraction. The results of electrophoretic analysis of these preparations show that the livetin fraction is a fairly complex mixture.

Electrophoresis

The Longsworth modification (6) of the Tiselius apparatus has been used for electrophoretic analysis. Unless otherwise stated, the buffer solution consisted of 0.0253 M disodium phosphate, 0.00133 M monopotassium phosphate, and 0.0218 M sodium chloride, pH 7.95, and ionic strength 0.099. A field strength of about 6 volts per cm. was employed in most cases for 180 minutes. Areas were measured under peaks of the descending side only and mobilities were estimated from the distance between the peak in question and the ϵ peak.

Methods for Preparation of Livetin

Method A—Unless otherwise specified, ether-extracted preparations were obtained as follows. The egg yolks were separated, washed in tap water, and then broken up. After adding 2 volumes of 0.85 per cent sodium chloride, the mixture was filtered through cotton and was shaken thoroughly with about 2 volumes of ether and allowed to stand overnight at room temperature. The clear aqueous layer was drawn off and again shaken with 2 volumes of ether. In this manner, a total of six ether extractions was carried out, and a clear aqueous solution of yolk components was obtained.

When the dissolved ether was removed from the aqueous solution by suction with a water pump, a precipitate appeared. This precipitate was centrifuged and retained for further study. The supernatant was dialyzed against the buffer used for electrophoresis. A small amount of precipitate formed during dialysis, but the solution could usually be clarified by centrifugation.

If the ether extraction was carried out incompletely, for example, by only one or two extractions, the precipitate mentioned above did not appear. There was some difficulty in securing solutions clear enough for electrophoresis, because an increase in turbidity usually took place after pumping off the dissolved ether and after dialysis against the buffer; only by prolonged centrifugation and filtering were clear solutions subsequently obtainable.

Method B—To prepare yolk proteins without the use of ether, the egg yolks were treated as described above and 1 volume of 0.85 per cent sodium chloride was added before filtration through cotton. The filtrate was then dialyzed against 0.85 per cent sodium chloride for about 1 week in the refrigerator. Fresh 0.85 per cent sodium chloride solution at room temperature was added morning and night. At the completion of dialysis, preparations have been found to contain about 12 per cent of ether-extractable solids and about 9 per cent of solids which were not dialyzable and not extractable with ether. At this stage a precipitate was present which contained little ether-extractable material, but did contain about one-fourth of the non-dialyzable, non-extractable solids. The precipitate was removed by centrifugation.

The supernatant solution was then diluted with 0.5 volume of distilled water to lower the molarity of sodium chloride to about 0.1, and the solution was adjusted with hydrochloric acid to pH 6.0 to 6.2 as measured by the glass electrode. A voluminous, yellow, flocculent material separated and rose to the top of the suspension. This material contained about 70 per cent ether-extractable solids and was difficult to separate completely from the solution. By allowing the suspension to stand in the cold (6°) a few hours in a separatory funnel, the lower phase could be drawn off and clarified by filtration, first through a porcelain filter, then through a Seitz sterilizing pad. The filtrate which contained about 3 per cent non-dialyzable solids and 1.3 per cent ether-extractable material was usually clear enough for electrophoresis. When the filtrate was not satisfactory, it could be adequately clarified by dialyzing overnight in 10 volumes of distilled water. Subsequent filtration through a Seitz sterilizing pad resulted in a clear solution.

Precipitate by Method A—The precipitate, which formed when the dissolved ether was removed from the thoroughly extracted aqueous solution of yolk, has the following properties. (1) After being washed repeatedly in 0.85 per cent sodium chloride solution, it dissolved in 0.85 per cent salt solution on the addition of an excess of ether. (2) The solution was slightly opalescent and was stable at room temperature for months when excess ether was present. (3) When the solution was stored at temperatures below 20°, a precipitate appeared which dissolved slowly when the temperature was raised to 20–25°. (4) When the precipitate of Method A was washed five times with distilled water and dried from the frozen state, a white fluffy solid resulted which contained[1] nitrogen 9.51, phosphorus 1.16, sulfur 0.45, and ash 0.87 per cent. 39 per cent of the solid was extractable in cold 95 per cent ethanol. The ethanol-soluble portion was soluble in ether and when dried was a white waxy solid. The residue after alcohol extraction was not soluble in 0.85 per cent salt solution saturated with ether. (5) When 0.1 M sodium caprylate was added to a 1 per cent solution of the precipitate of Method A in 0.85 per cent salt solution and excess ether, a solution resulted which could be cooled to 6° and from which the ether could be removed without the appearance of a precipitate. However, dialysis in the buffer used for electrophoresis resulted in precipitation. (6) A stable solution suitable for electrophoresis at 1° was prepared as follows: 30 ml. of a 1 per cent solution of the precipitate of Method A in 0.85 per cent salt solution saturated with ether were repeatedly shaken with 45 ml. of ether which had been used to extract 45 ml. of a mixture of 1 part of yolk in 2 parts of 0.85 per cent salt solution. When the dissolved ether was removed by vacuum from the aqueous layer, a solution was obtained which contained 0.66 per cent of non-dialyzable solids after dialysis and clarification for electrophoresis.

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[1] These analyses were carried out by Mr. C. A. Kinser.

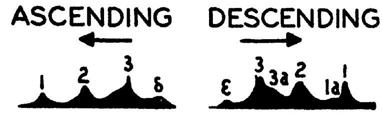
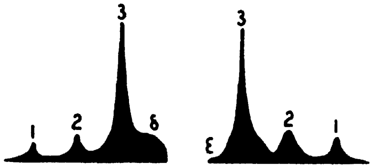


FIGURE 1



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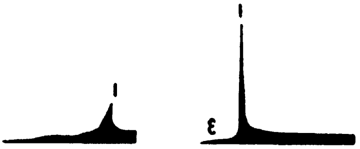
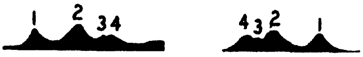


FIGURE 3



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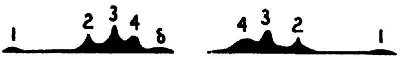

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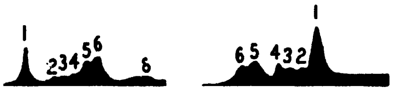

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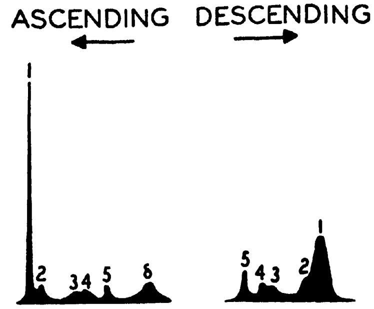

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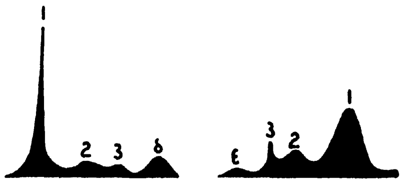

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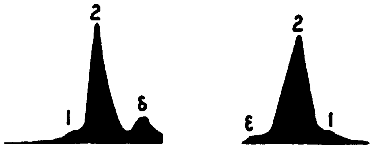

FIGURE 9

Fig. 1. Livetin fraction prepared by extraction six times with ether.
Fig. 2. Livetin fraction prepared by extraction one time with ether.
Fig. 3. Precipitate of Method A treated with ether extract of yolk.
Fig. 4. Livetin fraction prepared according to Method B without ether extraction.
Fig. 5. Material of Fig. 4 after ether extraction.
Fig. 6. Hen plasma.
Fig. 7. Egg white.
Fig. 8. Allantoic fluid from 13 day fertile eggs.
Fig. 9. Livetin fraction, precipitate in 18 per cent ethanol.

Results of Electrophoretic Analysis of Livetin Preparations

An electrophoretic pattern of crude livetin prepared according to Method A is given in Fig. 1. Three principal peaks (*1*, *2*, and *3*) can be seen with respective mobilities of -7×10^{5}; -4.4×10^{5}, and -2.0×10^{-5} sq. cm.

per volt-second (*cf.* Table I). A minor peak (*1a*) usually is found with a mobility of -6.4×10^{-5} sq. cm. per volt-second. The slowest peak appears to consist of two components: *3*, with a mobility of about -2×10^{-5}, and *3a*, with a mobility of about -2.6×10^{-5} sq. cm. per volt-second.

A preparation of livetin which was extracted only once with 1 volume of ether and clarified by several centrifugations and filtrations is shown in Fig. 2. An increased amount of the slower component of the slowest peak can be seen as a sharp peak (*3*) with a mobility of -1.7×10^{-5} sq. cm. per volt-second. The presence of the other more rapid component (*3a* of Fig. 1) is suggested by skewness at the base of the peak. Further extraction with ether removed the material of the sharp peak and left the other components present as they appear in Fig. 1.

TABLE I

Results of Electrophoretic Analyses of Yolk Fractions and Related Materials

The numbers in the column headings refer to the labelled peaks in Figs. 1 to 9. All mobilities are negative, since migration was anodic in every instance.

Fig. No.	Material	Mobility × 10⁵, sq. cm. per volt-sec.						Areas, per cent					
		1	*2*	*3*	*4*	*5*	*6*	*1*	*2*	*3*	*4*	*5*	*6*
1	Livetin, Method A, extracted 6 times	6.99 6.44	4.42	2.02 2.64				17.3	32.7	50.0			
2	Livetin, Method A, extracted 1 time	7.55	4.64	1.71				12.0	21.2	67.0			
3	Ppt. of Method A, ether solution treated	1.76						100					
4	Livetin, Method B, not extracted	5.27	2.71	1.35	0.64			35.0	39.0	26.0			
5	Livetin, Method B, after extraction	14.96	7.29	4.54	2.51				21.3	40.4	38.4		
6	Hen plasma	6.42	4.85	4.23	3.44	2.82	1.88	53.3	18.3			28.3	
7	Egg white	4.0	3.4	2.2	1.8	1.2		71.1		17.2		11.7	
8	Allantoic fluid, 13th day	6.82	3.45	1.98				69.7	18.6	11.7			
9	Livetin, 18% EtOH ppt.	4.86	2.65					2.2	97.8				

When the precipitate of Method A was dissolved by means of an ether solution of the ether-extractable substances of yolk, as described above, a solution resulted which yielded the electrophoretic pattern shown in Fig. 3. There appears to be chiefly one component with mobility of $-1.8 \times$

10^{-5} sq. cm. per volt-second. This is apparently the material of the sharp peak of Fig. 2 which was rendered insoluble by thorough extraction with ether.

The analysis of material prepared according to Method B, including dialysis against distilled water, is shown in Fig. 4. Three principal peaks are seen: *1* and *2* have mobilities close to those of the two slower peaks (*2* and *3a*, Fig. 1) of fully extracted yolk, the third peak (*3* and *4*) is somewhat slower than any seen in Fig. 1. Ether extraction of such preparations removes nearly half of the non-dialyzable solids and leaves a preparation with the pattern seen in Fig. 5. The three peaks (*2*, *3*, and *4*) corresponding to the three peaks (*1*, *2*, and *3a*) of Fig. 1 are seen, but there is no suggestion of the sharp peak (*3*, Fig. 2) of slow mobility which characterizes the incompletely extracted preparation.

Livetin prepared according to Method A was also extracted with cold ethanol and ether by the technique of Zeldis, Alling, McCoord, and Kulka (7) and was examined electrophoretically. A pattern very similar to Fig. 5 resulted in which there was no evidence of the slow component giving the sharp peak.

Comparison of Livetin Preparations with Other Fluids

Electrophoretic analyses were also made of hen plasma (Fig. 6), egg white (Fig. 7), and allantoic fluid from fertile eggs incubated 13 days (Fig. 8). It will be noted that the fastest peak (*1*, Fig. 6) in hen plasma, presumably serum albumin, has a mobility similar to that of the fastest peak (*1*, Fig. 1) in the livetin preparations. The two large peaks (*5* and *6*, Fig. 6) moving most slowly in hen plasma have a mobility similar to that of the slowest peak (*3* and *3a*, Fig. 1) in livetin preparations. The most rapid peak of Fig. 7 (*1*) is ovalbumin according to the work of Longsworth, Cannan, and MacInnes (8), and it appears to move at a somewhat slower rate than the fastest livetin peak (*1*, Fig. 1). The mobilities of the fastest and slowest components (*1* and *3*, Fig. 8) of 13 day allantoic fluid are similar to these of the corresponding components of livetin (*1* and *3*, Fig. 1). Further work comparing the components of egg yolk with those of hen serum is in progress.

Fractionation Experiments with Ethanol

Preliminary studies of the precipitation of livetin components with ethanol in the cold have been carried out. The livetin was dialyzed against sodium chloride-phosphate buffer of the following composition: 0.0095 M monopotassium phosphate, 0.0022 M disodium phosphate, 0.23 M sodium chloride, pH 7.2, ionic strength 0.246. Ethanol was added at 0° to a concentration of 18 per cent by volume. The precipitate was washed with the buffer containing 18 per cent ethanol, then dissolved in 0.85 per cent sodium chloride solution, and dialyzed against the electrophoresis buffer. The electrophoretic pattern is shown in Fig. 9. It was found that the component of slow mobility (*3a*) of Fig. 1 was recovered with greater than 95 per cent homogeneity. However, the other two principal components of Fig. 1 (*1* and *2*) were not well separated by alcohol precipitation under the conditions of the few experiments performed.

DISCUSSION

Since the name livetin has been applied to the material remaining in solution after lipovitellin has been precipitated from ether-extracted egg yolk by lowering the salt concentration, it is not surprising that electrophoretic analysis shows livetin to be not one substance but a group of substances. The materials present in the livetin preparation show electrophoretic mobilities resembling components of hen plasma. Of the three peaks seen in Fig. 1 the fastest (*1*) has, within the limits of measurement, the mobility of hen serum albumin (*1*, Fig. 6). The second peak (*2*) compares with the slowest of the β-globulins, and the slowest peak (*3*) has a mobility not much different from that of the second (*6*, Fig. 6) of the two large peaks which characterize hen serum (9, 10).

That there is a transfer of proteins from the plasma of laying hens to the yolks of their eggs is evidenced by the passive transfer of antibodies. Jukes, Fraser, and Orr (11) demonstrated in 1934 that diphtheria antitoxin was found in the livetin fraction of egg yolk. The literature on antibodies in yolk has been reviewed recently by Brandly, Moses, and Jungherr (12).

Since lipovitellin itself has been shown to be a constituent of the plasma of laying hens, it has been inferred that this component is merely transferred to the yolk in the ovary, its site of formation being elsewhere (13).

The passage of large molecules from plasma to yolk during egg formation seems possible from anatomical considerations (14). The yolk of the avian egg is formed in the ovary, the yolk being enveloped by follicular epithelium. Although it is possible that the follicular epithelium plays a synthetic rôle, it seems likely that not all of the yolk volume is synthesized there because of volume-rate considerations (15).

The precipitate of Method A, described above, is of a composition which indicates its relationship to the lipovitellenin of Fevold and Lausten (5). Since no reports of the homogeneity of either lipovitellin or lipovitellenin have been seen, it is difficult to come to conclusions about the identity of the precipitate of Method A with lipovitellenin. Although the amounts of alcohol-extractable substances and nitrogen are not far from those reported for lipovitellenin, the phosphorus and sulfur values (in per cent) are somewhat different.

	N	P	S	Alcohol-extracted lipide
Lipovitellenin (Fevold and Lausten (5))	9.9–10.2	1.5–1.7	0.59–0.61	36–41
Ppt. by Method A	9.51	1.16	0.45	39

SUMMARY

1. Clear preparations of the livetin fraction of the yolk of hen's eggs were produced when the extraction with ether was adequate. Electrophoretic examination of this material at pH 7.95 revealed three principal peaks.
2. When the dissolved ether was removed from well extracted livetin fractions, a precipitate appeared of a composition which suggested a lipoprotein containing about 39 per cent ethanol-extractable material.
3. A method of preparation of livetin fractions which does not make use of ether was worked out. Dialysis and adjustment of salt content and pH were employed. Electrophoretic examination of this material gave results similar to those with livetin fractions prepared by ether extraction.
4. Electrophoretic analyses of hen plasma, egg white, and allantoic fluid were carried out under comparable conditions. The mobility of hen plasma albumin and two of the globulins was similar to those of the three livetin fraction peaks.
5. Preliminary attempts directed toward separation of components of the livetin fraction by ethanol precipitation are reported. The slowest component by electrophoresis was obtained in a form essentially free from the other observed components.

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THE UTILIZATION OF GLYCINE IN THE BIOSYNTHESIS OF HEMOGLOBIN*

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Isotopic studies have demonstrated that glycine is used in the biosynthesis of hemoglobin. Glycine has been tagged in one of three ways: (*a*) with N^{15} in the amino group (1–3), (*b*) with C^{14} in the α-methylene carbon position (4), and (*c*) with C^{14} in the carboxyl group (5). When any of these three forms of glycine is fed to animals, the isotope is incorporated into hemoglobin, but interesting differences have been observed in the distribution of the isotope between the pigment and protein portions of the molecule. For instance, Shemin and Rittenberg, using glycine labeled with N^{15}, found greater concentrations of the heavy nitrogen in hemin than in red blood cell protein (2). Similarly, Altman and his associates observed that when the methylene carbon of glycine is tagged 7 to nearly 10 times as much C^{14} appear in hemoglobin protoporphyrin as in globin (4). On the other hand, recent observations in this laboratory have shown that when glycine containing C^{14} in the carboxyl group is administered the radioactive carbon is synthesized into globin but cannot be demonstrated in protoporphyrin (5).

The experiments to be described in this report were designed (1) to confirm the demonstration that the carboxyl carbon of glycine is used for the biosynthesis of globin but not protoporphyrin, (2) to determine whether globin within the red cell participates in protein interchange, and (3) to discover whether coproporphyrin I isolated from the urine and feces after the tagged glycine was fed would contain C^{14} even if the hemoglobin protoporphyrin did not.

Materials and Methods

Two healthy dogs and one rat were fed glycine tagged with C^{14} in the carboxyl group.[1]

Dog I, male, weighed 12 kilos. By removing 150 to 200 ml. of blood from the femoral artery on each of several days, this animal's packed

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* Supported by a grant from Mr. John Mosby and one established in memory of Mr. Nathan Greenberg.

[1] The authors are indebted to Dr. R. B. Loftfield of the Massachusetts Institute of Technology for the synthesis of the labeled glycine.

erythrocyte volume was reduced from 51 to 29 per cent. 100 mg. of the labeled glycine were then given by stomach tube in divided doses on 3 successive days; the total radioactivity administered amounted to 5×10^7 counts per minute, assayed by a procedure described elsewhere (5, 6). During these 3 days, water was allowed *ad libitum* but all food was withheld. Thereafter, the stock ration of Purina dog chow was again fed. At intervals until the experiment was terminated on the 153rd day, 10 ml. samples of blood were obtained for analysis. Crystalline protoporphyrin IX dimethyl ester (7) and globin (8) were isolated for determination of radioactivity (5, 6).

Dog II, female, weighed 10 kilos. The packed red blood cell volume was reduced from 53 to 24 per cent by serial bleeding. The labeled glycine was again given by stomach tube in three divided doses within a period of 27 hours; the total dose was 50 mg. and the total radioactivity amounted to 1.7×10^7 counts per minute. Food was withheld during the period of administration. Blood was collected at intervals in 10 ml. samples as in the previous experiment for the same determinations, but observations were stopped at the end of 110 days. In addition, urine and feces were collected during the first 15 days following administration of the glycine and extracted for coproporphyrins (9). After purification and identification (10) coproporphyrin I was tested for radioactivity with a nucleometer.[2]

One white rat weighing 100 gm. was kept on a protein-free diet (2) for several days and then given by stomach tube, in four divided doses over 3 days, 200 γ of the labeled glycine (total radioactivity 2.1×10^5 counts per minute). 20 days later the animal was bled to death. From the 4.6 ml. of blood obtained, crystalline protoporphyrin IX dimethyl ester was isolated for determination of its radioactivity. Measurement was also made of the radioactivity in the remaining cell protein.

All samples were assayed without resorting to conversion to carbonate. Satisfactory checks were obtained in pilot experiments in which samples were assayed both as carbonate and as untreated material. All samples were measured in triplicate and values reported are averages, the spread of which is included in the errors assigned.

Results

No radioactivity could be demonstrated in the protoporphyrin isolated from the blood of any of the three animals. The activity in the globin obtained from the dogs was at a maximum on the 1st day determinations were performed, the 23rd and 13th days, respectively (Fig. 1 and Table I). The concentration remained relatively constant for 77 days in Dog I and until about the 66th day in Dog II. Thereafter, the radioactivity fell rather abruptly to low levels. In Fig. 1 are plotted the results obtained for Dog I. When mathematical analysis of this curve was made in the manner described by Shemin and Rittenberg (2) to estimate the average

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[2] The authors are indebted to Professor A. I. Lansing, Department of Anatomy, Washington University School of Medicine, for permission to use the nucleometer, a product of the Radiation Counter Laboratories, Chicago, Illinois. The nucleometer was calibrated with standard samples of labeled glycine assayed previously with the standard, thin-walled Geiger-Müller tube (5).

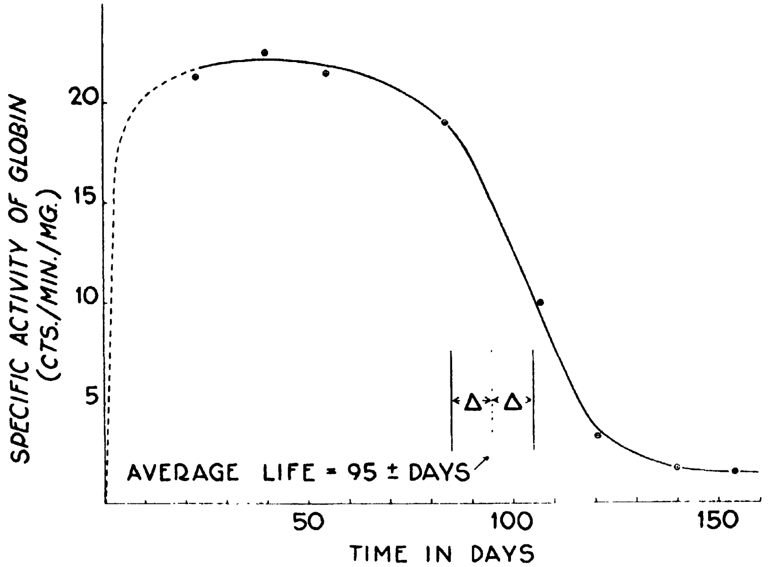

FIG. 1. Radioactivity in globin prepared from blood of Dog I after oral administration of glycine tagged with C^{14} in the carboxyl position. Δ represents half the period required for half the cells to die.

TABLE I

Radioactivity in Hemoglobin Protoporphyrin and Globin of Dog II after Oral Administration of Glycine Containing C^{14} in Carboxyl Group

Time	Radioactivity, counts per min. per mg. in	
	Protoporphyrin IX dimethyl ester	Globin
days		
13	<0.1	11.2 ± 0.6
33	<0.1	10.1 ± 0.5
47	<0.1	11.7 ± 0.4
66	<0.1	9.5 ± 0.5
80	<0.1	3.9 ± 0.1
93	<0.1	3.7 ± 0.2
110	<0.1	3.2 ± 0.2

survival time of red blood cells of the dog, a figure of 95 ± 5 days was obtained. The period in which half of the red cells was destroyed extended from the 85th to the 105th day, as shown in Fig. 1. The average survival time of the red blood cells for Dog II, determined in the same manner, was 75 ± 5 days. The cell protein from 4.6 ml. of the rat's blood contained 1.5 ± 0.5 counts per minute per mg., a value reasonably close to that to be expected from the initial dose of glycine administered, in light of the experience with uptake into globin in the dogs.

From the urine and feces collected for 15 days following the last administration of glycine to Dog II, 310 γ of crystalline coproporphyrin I tetramethyl ester were isolated. No radioactivity was observed.

DISCUSSION

These results confirm the previous demonstration that in the biosynthesis of hemoglobin the carboxyl carbon of glycine is incorporated into globin but not into protoporphyrin. Since other work has shown that both the amino nitrogen (1–3) and the methylene carbon (4) of glycine are introduced into protoporphyrin during its formation, it would appear that only the $—CH_2\cdot NH_2$ group of glycine becomes a part of protoporphyrin. If the whole of the glycine molecule is involved in the synthesis of protoporphyrin, CO_2 from the carboxyl group must be split off in the process.

On the other hand, since the amino nitrogen (2) and the methylene carbon (4) atoms as well as the carboxyl carbon are incorporated into globin, it seems likely that glycine as a whole is utilized for globin synthesis. The data available do not permit any conclusion as to whether CO_2 detached from glycine can be used for globin formation (11).

When the radioactivity of globin is plotted against time, the shape of the resulting curve is very similar to that obtained for protoporphyrin when the concentration of N^{15} is plotted against time (2). This result indicates that the globin of intact erythrocytes remains in the red cells without participating in protein interchange until the erythrocyte is destroyed.

The radioactivity of the globin fraction did not fall off with time to as low a value as might be expected on the basis that C^{14} from degraded globin was not reutilized for globin synthesis. Insufficient data are at hand to decide to what extent such reutilization occurred. It seems unlikely, however, that simple reutilization of glycine from degraded globin can be reconciled with results such as those shown in Fig. 1. Miller, Robscheit-Robbins, and Whipple have reported evidence indicating that globin contributes to the "protein pool" of the body, and that this "protein pool" in turn is used for the formation of new hemoglobin (12). When hemoglobin is injected intraperitoneally into dogs made both anemic and hypoproteinemic, new hemoglobin is formed. The conditions of these experiments, however, were quite different from those reported in the present paper. The two dogs used were not anemic at the conclusion of the experiment and had been fed a stock ration known to be nutritionally adequate.

The average times of survival of the red cells in Dogs I and II, as measured by the curves of radioactivity in globin, were 95 ± 5 days and 75 ± 5 days, respectively. Hawkins and Whipple estimated the life span of dog red cells to be about 124 days (13). These workers forced great numbers of new red cells into the circulation of four bile fistula dogs by blood destruction or blood withdrawal; as a consequence, the bile pigment output fell and did not rise again for 112 to 133 days. This interval was regarded as an indication of the survival time for the red cells. No conclusion is drawn regarding the difference between these figures and the ones calculated from the globin curves. More observations would be necessary to establish a more accurate average figure and the expected limits of variation.

Since available evidence indicates that coproporphyrin I is formed as a by-product during the synthesis of protoporphyrin IX (type III) for hemoglobin formation, and since hemoglobin protoporphyrin did not assimilate any radioactivity from the C^{14} of the carboxyl-tagged glycine, it is not surprising that the coproporphyrin I isolated from Dog II likewise possessed no radioactivity. Other experiments, furthermore, have shown that when coproporphyrin I is isolated from the feces of an animal fed glycine tagged with N^{15} the fecal coproporphyrin I as well as hemoglobin protoporphyrin IX (type III) contains heavy nitrogen in relatively high concentration (14). These results provide additional evidence in favor of the hypothesis that coproporphyrin I is a by-product of protoporphyrin IX formation.

SUMMARY

1. In the biosynthesis of hemoglobin, the carboxyl carbon is incorporated into globin but not into protoporphyrin.

2. Globin within the intact erythrocyte does not participate in protein interchange. No evidence was obtained to indicate that the C^{14} derived from the carboxyl carbon of glycine and synthesized into globin was reutilized for hemoglobin formation.

3. Coproporphyrin I isolated from the feces of one animal during a 15 day period, following the administration of carboxyl-tagged glycine, contained no radioactivity. Reference was made to other experiments in which coproporphyrin I was isolated from the feces of an animal fed N^{15}-tagged glycine; both the fecal coproporphyrin I and the hemoglobin proto-

porphyrin IX (type III) contained the isotope. These observations support the concept that coproporphyrin I is a by-product of protoporphyrin IX formation.

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CHEMICAL PREPARATION OF HOMOGENTISIC ACID*

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Homogentisic acid (2,5-dihydroxyphenylacetic acid), an important intermediate in the metabolism of tyrosine and phenylalanine, is not readily available, nor does a survey of the literature reveal a convenient method for its chemical preparation.

The syntheses of Baumann and Fränkel (1), Osborne (2), and Neubauer and Flatow (3), although useful as proof of structure, are not suitable as preparative methods. Hahn and Stenner (4) developed a synthesis based on the ozonization of dibenzoylallylhydroquinone which yielded homogentisic acid from hydroquinone in six steps with a 26 per cent yield. In the course of studies on the reactions of ketene diethyl acetal, McElvain and Cohen (5) noted that acid hydrolysis of the reaction product of benzoquinone with ketene acetal yielded homogentisic acid. The procedures involved in these two methods make their use inconvenient as preparative methods in most laboratories.

Recent developments in the application of the Willgerodt reaction to the synthesis of arylacetic acids from aryl methyl ketones (6, 7) and the commercial availability of the dimethyl ether of hydroquinone[1] make feasible the synthesis of the dimethyl ether of homogentisic acid (2,5-dimethoxyphenylacetic acid) from 2,5-dimethoxyacetophenone. Hydriodic acid, or hydriodic acid and red phosphorus, has been used previously to demethylate the dimethyl ether of homogentisic acid (1, 2). Although this reagent does bring about demethylation of this ether, its use in our hands has been unsatisfactory. We have found 2,5-dimethoxyphenylacetic acid to be easily demethylated with 48 per cent hydrobromic acid, a reagent which has been used to demethylate guaiacol (8). The desired product was thus obtained readily in good yield and in good condition.

EXPERIMENTAL

2,5-Dimethoxyacetophenone—This compound has been prepared previously by Klages (9) and by Kauffmann and Beisswenger (10) by the Friedel-Crafts reaction in yields of about 50 per cent. As our procedure has been found to give the desired compound in greater yield, we are presenting its preparation in detail.

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* A brief report of this work was made at the Twenty-sixth annual meeting of the Virginia Academy of Science, Roanoke, May 7, 1948.

[1] Obtained from the Tennessee Eastman Corporation, Kingsport, Tennessee.

A mixture of 150 ml. of dry carbon disulfide and 86 gm. (0.64 mole) of anhydrous, powdered aluminum chloride was cooled to 5° in a 1 liter, 3-necked flask equipped with a motor stirrer, reflux condenser, and dropping funnel. A cooled solution consisting of 44 gm. (0.56 mole) of acetyl chloride, 150 ml. of dry carbon disulfide, and 68 gm. (0.49 mole) of 1,4-dimethoxybenzene was added from the dropping funnel to the aluminum chloride with stirring, during the course of 1½ hours, in a hood. The mixture was stirred at 5° for an additional 3 hours, and then allowed to warm up and remain at room temperature for 17 hours. The stiff, red tar was hydrolyzed with a mixture of 40 ml. of concentrated hydrochloric acid and 1200 ml. of crushed ice. The carbon disulfide layer was separated, and the water layer extracted once with 100 ml. of carbon disulfide. The combined carbon disulfide layers were treated twice with dilute sodium hydroxide, once with water, and then with anhydrous calcium chloride to dry. The carbon disulfide was removed by distillation on a steam bath and the residue distilled in two fractions at 10 mm. pressure. The first fraction, consisting of unchanged dimethoxybenzene and some dimethoxyacetophenone, distilled at 95–140° and weighed 10 gm. The second fraction of nearly pure dimethoxyacetophenone distilled at 141–144° and weighed 61 gm. (69 per cent of the theoretical yield or 81 per cent based on unrecovered dimethoxybenzene). This material was used in the following step. Use of the undistilled dimethoxyacetophenone is practicable but necessitates more elaborate purification of the dimethoxyphenylacetic acid.

2,5-Dimethoxyphenylacetic Acid—The procedure of Schwenk and Bloch (7) has been modified so that the yield was increased from 28 to 64 per cent.

A mixture of 57 gm. (0.32 mole) of 2,5-dimethoxyacetophenone, 15.5 gm. (0.48 mole) of sulfur, and 42 gm. (0.48 mole) of morpholine was placed in a 1 liter round bottom flask and refluxed in a hood for 7 hours. The mixture was cooled to room temperature and 240 ml. of 95 per cent ethanol and 105 ml. of 50 per cent sodium hydroxide were added. After this mixture had been refluxed for 45 hours, the ethanol was removed by distillation and 200 ml. of water were added to the warm mixture. This mixture was heated to boiling, and the excess sulfur filtered off. After being cooled, the filtrate was placed in an efficient hood (or out of doors) and concentrated hydrochloric acid (about 220 ml.) was added, slowly and with constant stirring, until the filtrate was acid to Congo red paper. The mixture was then cooled overnight at 5° and the precipitated acid was collected by filtration. The crude acid was purified by dissolving it in about 2 liters of boiling water, treating with Darco, filtering, and cooling overnight at 5°. The cream-colored acid weighed 40 gm. (64 per cent), melted at 121–123°, and was used in the following step without further purification. An additional recrystallization from water gave pure white needles melting at 123–124°.[2]

Homogentisic Acid and Its Lactone—Demethylation of 2,5-dimethoxyphenylacetic acid with HBr yielded mixtures of the lactone and the free acid, as did demethylation with HI. We have used conditions giving predominantly the lactone, which can be readily purified. Addition of NaOH in the presence of Na_2SO_3 allowed opening of the lactone ring without occurrence of the intense darkening and rapid oxidation of homogentisic acid which took place when the lactone was converted in the presence of air with alkali alone.

10 gm. of 2,5-dimethoxyphenylacetic acid and 75 ml. of 48 per cent hydrobromic acid[3] were refluxed gently (in a hood) for 4 hours. The dark solution was then evaporated to dryness *in vacuo* on a boiling water bath. The residue was washed once with chloroform to remove traces of unchanged starting material.[4] The deeply colored lactone was then dissolved in about 400 to 500 ml. of boiling water, treated at the boiling point with about 1 gm. of decolorizing charcoal (Darco), and filtered hot.[5] To the warm filtrate, which was almost colorless, 2 gm. of Na_2SO_3 (anhydrous, Merck reagent) were added for each 100 ml. of filtrate. The solution was made distinctly alkaline to litmus (pH 10 with Fisher Alkacid test paper) with 10 per cent NaOH. After stirring and standing for about 5 minutes to insure complete conversion of the lactone, the solution was cooled to room temperature and made definitely acid to Congo red paper (pH 2 with Alkacid paper) with 10 per cent H_2SO_4. The solution was then concentrated *in vacuo* on a warm water bath to about 50 ml. Any precipitated material was redissolved with a minimal amount of water and the cooled solution, which should be acid to Congo red, was subjected to a 9 hour continuous extraction with peroxide-free ether. The ether was removed under reduced pressure. Homogentisic acid obtained in this way was light tan, melted at 146–148°, and weighed 8 gm. (93 per cent).

Additional purification was obtained by recrystallization in the following manner. The product above was dissolved in a small amount of hot absolute alcohol, and 75 to 100 ml. of a (1:1) chloroform-petroleum ether mixture were added. The solution was filtered free of any insoluble material while warm and still clear. More petroleum ether-chloroform mixture was then added to the warm filtrate until slightly turbid. On cooling, 4.3 gm. (50 per cent) of a white product, m.p. 148–149°, were obtained. Colored material remained in the filtrate. From this filtrate an additional 1.3 gm. (15 per cent) melting in the same range, but slightly off white in color, were obtained by the further addition of chloroform-petroleum ether. An additional 1 gm. (11.8 per cent) of slightly less pure material (m.p. 146–148°) was obtained by concentration of the filtrate and addition of chloroform-petroleum ether.

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[2] Melting points reported in this paper are uncorrected.

[3] Baker's, C. P., was used without purification.

[4] Although deeply colored, the residue was almost pure lactone, as indicated by its melting point (185–187°, literature 189–190°), and represented almost complete conversion of the dimethyl ether.

[5] If the pure lactone is desired, it may be obtained by cooling the decolorized filtrate. The pure white lactone (m.p. 188–189°) crystallized in 69 per cent of the theoretical yield from such filtrates on cooling and can be readily recrystallized further from boiling water if necessary.

No depression of the melting point was observed when the synthetic preparation was mixed with homogentisic acid isolated from the urine of human alkaptonuria (11) or from the urine of rats made alkaptonuric experimentally (12).

SUMMARY

1. A three-step chemical preparation of homogentisic acid in good yield is described.

2. 2,5-Dimethoxyacetophenone was prepared from 1,4-dimethoxybenzene and was converted to 2,5-dimethoxyphenylacetic acid which was demethylated with hydrobromic acid.

3. The lactone of homogentisic acid may be isolated as an intermediate if desired.

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PHYSICOCHEMICAL STUDIES ON LYMPH AND LYMPHOCYTE EXTRACTS FROM NORMAL AND STIMULATED LYMPHATIC TISSUE*

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Ehrich and Harris (1) have demonstrated that, following the injection of antigen into the feet of rabbits, extracts of popliteal lymph nodes and the lymph emerging from them contained antibodies earlier and in concentrations higher than in the blood serum, and they suggested that the local lymphatic system was the site of formation of those antibodies. Further study (2) showed that the lymphocytes contained in the lymph were richer in antibody than was the lymph plasma surrounding them, especially at the time of the greatest rate of formation of antibodies. The evidence presented was consonant with the concept that the lymphocyte may be the site of final synthesis of antibodies following the injection of antigens into the tissues.

At the same time, Dougherty, Chase, and White (3), in an adaptation of the work of McMaster and Hudack (4), showed that following the subcutaneous injection of an antigen into the mouse a lymphocyte-rich cell suspension prepared from pooled spleens and lymph nodes of those animals contained antibodies to the antigens injected.

The present paper reports electrophoretic and ultracentrifugal studies on lymph and lymphocyte extracts from rabbit lymph nodes both before and 5 days after injection of antigen into tissue drained by that node.

EXPERIMENTAL

Chinchilla rabbits weighing about 2000 gm. were injected in each hind foot-pad with 0.2 cc. of a 5 per cent suspension of killed dysentery organisms (*Shigella paradysenteriae*). After 5 days the animals were anesthetized by the injection of sodium amytal. An incision was made on the medial aspect of the knee, the semitendinosus and semimembranosus muscles divided, and the efferent lymphatic vessel of the popliteal lymph node was exposed. A ligature was placed around the vessel and as much lymph as possible was drawn from it into a tuberculin syringe moistened

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* This study was aided by a grant from the Commonwealth Fund.

with a solution of sodium citrate. The lymph thus collected was freed of lymphocytes by centrifugation and stored in the frozen state.

The popliteal lymph nodes were then excised and used for the preparation of lymphocyte extracts as follows: For preparing lymphocytes the lymph nodes were minced in saline solution and the coarser particles were removed by slow centrifugation. The supernatant was then passed through a No. 30 stainless steel mesh to free the lymphocytes. The latter were collected by differential centrifugation from this suspension. The final sediment of clumps of cells from the node was suspended in 4 volumes of saline solution and the suspension was alternately frozen and thawed three times, at −70° and 30° respectively, or was thoroughly ground in a mortar. A final centrifugation was performed to clear the suspensions of insoluble débris. This extract of lymphocytes was kept in the refrigerator with a bacteriostatic agent (merthiolate, 1:10,000) until it was studied, since freezing or desiccation from the frozen state caused considerable precipitation.

The individual specimens of lymph and lymph node extracts were insufficient in volume for physicochemical study. Accordingly, the specimens examined by electrophoresis and ultracentrifugation were pooled from several animals.

Before these analyses all samples were dialyzed against a buffer of 0.02 M with respect to sodium phosphate and 0.15 M with respect to NaCl at pH 7.4.

The electrophoretic experiments were carried out in the Tiselius apparatus in a cell of 2 cc. capacity. Occasionally separated samples were removed for ultracentrifugal analysis. Sedimentation constants were determined in an air-driven vacuum ultracentrifuge (5), the sedimenting boundaries being recorded by the scanning method of Longsworth (6). The analyses were made at room temperature which was always 20°. Since the temperature of the rotor spinning at 48,000 R.P.M. rose about 1.2° per hour, it was possible to estimate the average temperature over any desired time interval during a run. All values of sedimentation constants were reduced to conditions of pure water at 20°.

Results

The difference between efferent clarified lymph from popliteal nodes of normal rabbits and that from popliteal nodes excised 5 days after injection into the foot-pad of 0.2 cc. of 5 per cent suspension of killed dysentery organisms is illustrated in the electrophoretic patterns of Fig. 1. After the injection of antigen the lymph produced a total pattern area (Table I) which was more than 3 times that for the normal, and corresponded to a protein concentration of approximately 2 gm. per cent. All of the components in the lymph obtained from animals which had been injected with antigen were larger in about the same proportions, as is seen from the percentage values recorded in Table I, except that Component 3 is relatively larger and Component 6 (with the mobility of γ-globulin) is relatively smaller.

Electrophoretic patterns of extracts of lymphocytes from two pools of normal popliteal nodes and from two pools of popliteal nodes draining the site of injection of antigen are shown in Fig. 2. There is much more protein per node and also an extra component of higher mobility (see Table I) in the extract of lymphocytes from stimulated animals, but the elevation is least in the component having a mobility of serum γ-globulin. This is in agreement with the published patterns of White and Dougherty (7) on normal animals and those injected with adrenal cortical steroids.

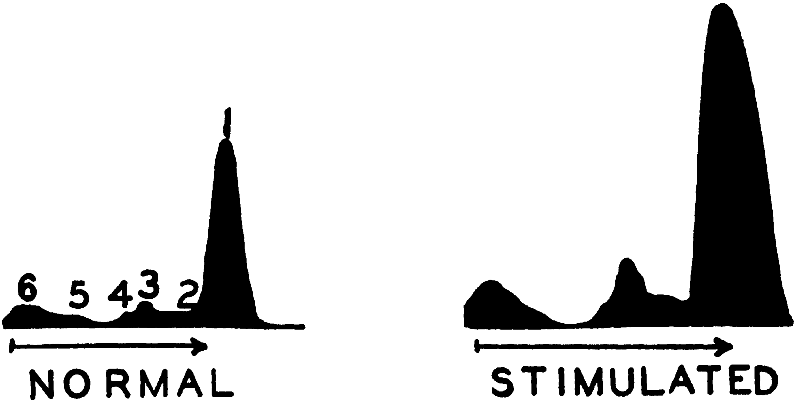

Fig. 1. Electrophoretic patterns of efferent lymph from normal popliteal nodes and from similar nodes after antigenic stimulation. The patterns are of the descending limb after 6.5 volts per cm. were applied 135 and 180 minutes respectively. The numbers refer to the components.

In the ultracentrifuge the normal lymphocyte extract appeared monodispersed (Fig. 3) and had a sedimentation constant of 4.7 Svedberg units. The extract from cells of stimulated nodes had a sedimentation constant of 3.6 Svedberg units, with a trace of material having sedimentation constants of about 5.3 Svedberg units. Component 6 (see Fig. 4), which was electrophoretically separated from clarified lymph 5 days after the introduction of antigen into the animal had a sedimentation constant of 7.0, which is the same as that of serum γ-globulin. No component with the sedimentation constant of serum γ-globulin was obtainable, however, from lymphocytes themselves, and in this respect we are not able to con-

TABLE I

Electrophoretic Data on Lymph and Lymphocyte Extracts from Normal and Antigenically Stimulated Rabbits

Specimen		Component No.								Average weight per node	Nodes per 3 cc. pool	Agglutinin titer*
		Fast	1	2	3	4	5	6	Total	*mg.*		
Lymph, normal	Composition†		200	15	20	10	10	35	290			
	%‡		69	6.9	6.9	3.4	3.4	12.1				
	Mobilities§		5.1	4.4	3.2	2.8	1.6	0.5				
Lymph after stimulation	Composition		705	40	85	20	50	90	990			768
	%		71.2	4.0	8.6	2.0	5.1	9.1				
	Mobilities		4.7	3.4	2.9	2.7	1.5	0.3				
Lymphocyte Extract 1, normal	Composition		75		260			85	420	105	10	
	%		18		62			20				
	Mobilities		5.0		3.4			0.5				
Lymphocyte Extract 2, normal	Composition		75		255			70	400	156	8	
	%		19		63			18				
	Mobilities		5.0		3.6			0.5				
Lymphocyte Extract 3, normal	Composition		20		55			50	125	85	16	
	%		16		44			40				
	Mobilities		4.4		3.4			0.6				
Lymphocyte Extract 1, after stimulation	Composition	40	75		290			115	520	684	6	1024
	%	8	14		56			22				
	Mobilities	6.8	5.1		3.5			0.5				
Lymphocyte Extract 2, after stimulation	Composition	85	100		280			75	545	716	6	2048
	%	16	18		52			14				
	Mobilities	6.3	4.7		3.4			0.5				
Lymphocyte Extract 3, after stimulation	Composition	70	45		165			60	340	730	6	2048
	%	21	13		48			18				
	Mobilities	5.6	4.3		3.5			0.1				
Normal serum	%		70	6		12		12				
	Mobilities		5.4	4.2		3.0		0.9				

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* The agglutinin titer is here given as an indicator of the concentration of the antibodies to one of the antigens of the dysentery organism. No measure of the total antibodies in the cell extract was available because of the nature of the antigen.

† Composition in planimeter units.

‡ Per cent of total area.

§ The mobilities are given in units of 10^{-5} sq. cm. per volt-second. All components are anodic.

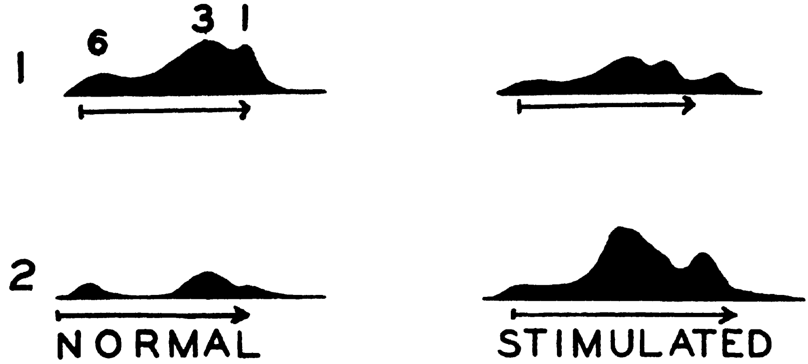

FIG. 2. Electrophoretic patterns of lymphocyte extract from nodes of normal and antigenically stimulated animals. Sets 1 and 2 show the variation in type of patterns obtained. The numbers on Set 1 refer to the components.

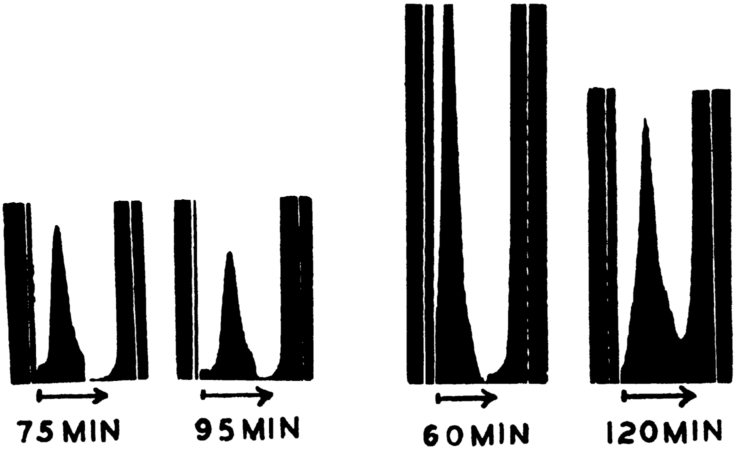

FIG. 3. Ultracentrifuge patterns of normal (left-hand) and stimulated (right-hand) lymphocyte extracts. The arrow butts indicate positions of the menisci. Speed, 48,000 R.P.M.; radius to the left reference line (white) 5.71 cm.; to the right reference line 7.29 cm.

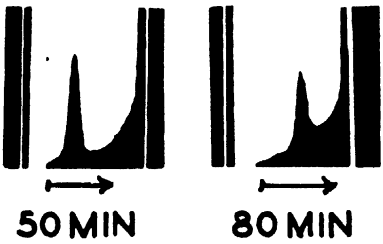

FIG. 4. Ultracentrifuge patterns of electrophoretically separated slow fraction from "stimulated" lymph. Speed, 48,000 R.P.M.

firm the assumption of Dougherty *et al.* (3) that the component with low electrophoretic mobility in lymphocyte extracts is identical with serum γ-globulin.

DISCUSSION

On comparing the electrophoretic patterns of lymph which has passed through normal and stimulated popliteal nodes, the characteristic finding would appear to be that all the components of lymph draining the site of deposition of antigen are found also in normal lymph, and except for total concentration differences, both materials closely resemble serum. The first question to be answered was whether the difference between the two fluids was due to an influx of blood serum into the lymphatic system. No experiments were performed to decide this point in the present study, since data obtained in earlier investigations could be used to show that the increased concentration of the components in lymph after the injection of antigen was not due to serum appearing in the latter. In those studies (1, 8) serologically different antigens had been injected into the opposite legs of rabbits, and at intervals thereafter the lymph of each leg was tested for antibodies not only to the antigen injected into that leg but to the antigen injected into the opposite leg as well. Simultaneous tests were made of antibody titers in the serum. The titer in each lymph sample to the heterologous antigen was never more than one-tenth that found to the same antigen in the serum. If we apply these data to serum proteins in general, it is possible to say that no more than 10 per cent of the constituents of lymph could be due to the inclusion of blood serum. Since the concentration of protein found in the lymph draining the site of antigen inoculation is approximately one-third that found in rabbit serum, it follows that at most only a small part of the proteins found in "stimulated" lymph, in excess of those found in normal lymph, could have resulted from the admixing of whole blood serum.

Attempts to determine the amount of antibody present in the lymph and lymphocyte extracts 5 days after the injection of antigen were not successful because the choice of so complex an antigen as a bacterial cell made quantitative measurements of the total antibody impossible. Of the several individual antibodies to antigens of the dysentery organism only two were measured, the agglutinin (9) and complement-fixing antibody to cytoplasmic particles of the organism (10). The quantities of both of these antibodies were estimated to be below the sensitivity of either electrophoretic or ultracentrifugal analysis.

As to the component with the mobility of albumin, some of the increase in this component over that found in normal lymph, probably about a fifth, is due to serum proteins. The remainder must be due to proteins which are the products of tissue destruction due to inflammation at the site of injection of the antigen. The rough similarity of the ratio of increase in the albumin-like to globulin-like components in stimulated lymph compared with the ratio of albumin to globulins in the blood serum may well be coincidental.

The electrophoretic data on extracts from washed lymphocytes show that the changes resulting from stimulation occur essentially in the components with higher mobilities and that there is little change in the component having the same mobility, 0.6 to 0.7 × 10^{-5} sq. cm. per volt-second, but not the same sedimentation constant of serum γ-globulin. White and Dougherty (7) have shown that there is a component in lymphoid tissue extracts with a mobility greater than that of serum albumin, which is greatly increased after the injection of adrenal cortical steroids (see (7) Fig. 1). The component with the same mobility as γ-globulin, however, does not appear to be increased in their records. In view of the fact that all antibodies described thus far have mobilities identical to that of serum γ-globulin (or similar to it, as in the case of the T component), it is evident that phenomena other than the production of protein with the same mobility as serum γ-globulin are occurring in lymphoid cells of nodes draining the site of injection of antigen. This view is consistent with the fact that no material having the sedimentation constant of serum γ-globulin is found in either normal or antigenically stimulated lymphocyte extracts, although there is a marked increase in the total protein (pattern area) after stimulation (Fig. 3). The low sedimentation constant of the antibody-containing lymphocyte extract would indicate that after the injection of antigenic materials there appears in the draining lymph node a significant amount of a substance either of low molecular weight, or of low density, such as lipoprotein. This material may be non-specific or may be a precursor to serum antibody, since its physical properties are different from those of serum γ-globulin.[1] The component with the mobility of γ-globulin found in lymph from antigenically stimulated popliteal nodes does have, however, the same sedimentation constant as does serum γ-globulin (7.0 × 10^{-13} cm. per second per unit field). Moreover, since this component in lymph, along with the others, is elevated by stimulation, it is possible that lymphocytes in the course of normal lysis in the lymph node would liberate serum γ-globulin as we know it, whereas on artificial lysis proteins having different physical properties might be found.

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[1] Many unrelated substances, such as pepsin digests of serum albumin or ragweed pollen extracts, behave electrophoretically as γ-globulin in the pH range usually employed.

SUMMARY

Saline extracts of lymphocytes, obtained from popliteal (regional) lymph nodes 5 days after the injection of killed dysentery organisms into the hind feet of rabbits, have been compared electrophoretically and in the ultracentrifuge with lymphocytes from uninjected legs. Components with higher mobilities were increased after the injection of the antigen, whereas the component with mobility similar to γ-globulin was not significantly increased. In the ultracentrifuge only components with sedimentation constants much lower than that of serum γ-globulin were found in either of the cell extracts.

Efferent lymph collected from the popliteal lymphatic system after the injection of the antigen showed an increase in all components over the normal. The γ-globulin electrophoretically separated from such lymph had the same sedimentation constant as the γ-globulin in blood serum.

It is a pleasure to acknowledge the most valuable technical assistance of Melba Costello and Trudy Schonberger.

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OCCURRENCE OF HYALURONIDASE INHIBITORS IN FRACTIONS OF ELECTROPHORETICALLY SEPARATED SERUM*

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An inhibitor to the action of hyaluronidase obtained from bovine testis has recently been described in human sera, and has been found to be associated with electrophoretically separated albumin (1). Another inhibitor of hyaluronidase has been described recently which inhibits streptococcal hyaluronidase and which gives evidence of being a true neutralizing antibody (2–4). Because of differences suggested between the natures of these two inhibitors by the studies referred to, the present study was undertaken to compare the antihyaluronidase activity found in electrophoretically separated samples of human serum when assayed with streptococcal and testicular hyaluronidase.

Methods and Materials

Sera were obtained from four normal subjects and from two patients with acute rheumatic fever. Electrophoresis of undiluted samples of fresh serum which had been dialyzed against Longsworth's barbiturate buffer at pH 8.6 yielded concentration gradients in the cell similar to those illustrated in Fig. 1. At the end of each run samples were removed from levels indicated by the horizontal dashed lines, and assayed by the mucin clot method.

The technique employed for the measurement of antihyaluronidase has been fully described elsewhere (4). In brief, it involves incubation of various dilutions of the serum or its fractions with a standard amount of hyaluronidase, and then as measurement of the residual, unneutralized enzyme. The latter is measured by its ability to depolymerize native hyaluronate to a degree such that the depolymerized substrate fails to demonstrate a property of the native hyaluronate; *i.e.*, the formation of a mucin-like clot on addition of acetic acid and protein.

Specimens of original sera and their fractions were tested for inhibition of hyaluronidase of both streptococcal and testicular origin. The streptococcal enzyme was prepared by concentration of a filtrate obtained after

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cultivation, in a protein-free bacteriologic medium, of a strain of group A hemolytic streptococci, selected for its ability to produce hyaluronidase (type 4). The testicular enzyme was generously supplied by the Wyeth Institute of Applied Biochemistry, Philadelphia.

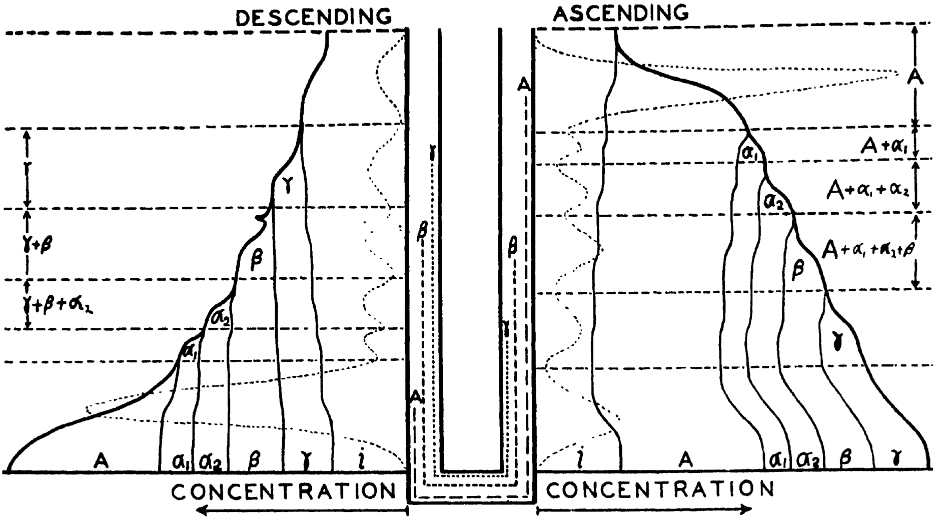

FIG. 1. Schematic representation of electrophoresis cell showing the way in which serum fractions were recovered at the end of a run. Positions of components and the way their concentrations vary throughout the cell are indicated. Buffer ion concentration is designated by *i*. Descending and ascending electrophoretic patterns which are a measure of the slope of the enveloping concentration curve or the change in total concentration (refractivity) throughout the cell are illustrated by the dotted curves.

TABLE I

Occurrence of Streptococcal Antihyaluronidase in Fractions of Sera of Two Patients with Acute Rheumatic Fever

Fraction	Subject I	Subject II
	units per ml.	*units per ml.*
A.	<5	<5
" + α_1	<5	<5
" + " + α_2	<5	<5
γ	960	1280
" + β	960	1920
Whole	1280	2240

* A. represents albumin; α_1, α_1-globulin; α_2, α_2-globulin; γ, γ-globulin; and β, β-globulin.

TABLE II

Occurrence of Two Inhibitors of Hyaluronidase in Various Fractions of Four Specimens of Serum

Subject	Fraction*	Protein†	Streptococcal		Testicular	
		gm. per 100 ml.	*units per ml.*	*units per gm.*	*units per ml.*	*units per gm.*
D. M.	A.	0.58	<5	0	1.9	325
	" + α_1	2.04	0	0	1.9	93
	" + " + α_2	2.48	0	0	1.9	77
	" + " + " + β	2.72	0	0	1.9	70
	γ	0.77	40	5,200	0	0
	" + β	1.40	40	2,860	0	0
	" + " + α_2	1.86	40	2,150	0	0
	Unseparated	4.0	40	1,000	1.9	48
T. H.	A.	1.64	<5	0	3.8	228
	" + α_1	2.61	0	0	2.5	96
	" + " + α_2	3.17	0	0	2.5	78
	" + " + " + β	3.50	0	0	2.5	72
	γ	1.19	240	20,000	0	0
	" + β	1.82	240	13,000	0	0
	" + " + α_2	2.22	240	11,000	0	0
	Unseparated	5.0	240	4,800	2.5	50
M. C.	A.	1.35	<5	0	10	740
	" + α_1	2.62	<5	0	6.3	238
	" + " + α_2	3.03	<5	0	6.3	207
	" + " + " + β	3.20	<5	0	5	312
	γ	0.5	30	6,000	0	0
	" + β	1.2	40	3,340	0	0
	" + " + α_2	1.65	40	2,430	0	0
	Unseparated	4.0	60	1,500	6.3	156
T. S.	A.	1.22	0	0	12.5	1025
	" + α_1	2.32	0	0	10	430
	" + " + α_2	2.93	0	0	6.3	213
	" + " + " + β	3.43	0	0	6.3	182
	γ	0.84	30	3,570	0	0
	" + β	1.5	40	2,670	0	0
	" + " + α_2	1.77	40	2,260	0	0
	Unseparated	4.1	60	1,460	7.5	183

* A. represents albumin; α_1, α_1-globulin; α_2, α_2-globulin; β, β-globulin; and γ, γ-globulin.

† Estimated from refractometric readings on a Zeiss interferometer.

Results

The first electrophoretic analyses were performed to determine the fraction which contained the inhibitor of the streptococcal enzyme mentioned above. Because, in our experience, the highest known concentrations of this inhibitor in serum occurred in patients with acute rheumatic fever (3, 5), the sera of two such patients were examined. The results of these analyses, as shown in Table I, indicated that all of the antistreptococcal inhibitor occurred in the γ-globulin fraction, although the original concentration of this antihyaluronidase in the serum was so high that a very small fraction of it could have been detected in other fractions, had it occurred.

Because of this clear electrophoretic differentiation between the antitesticular and antistreptococcal inhibitors, an analysis was carried out of the distribution of these inhibitors in the sera of four normal human subjects. The results of this analysis are shown in Table II. The results given show that, in agreement with the previous report in which the antitesticular factor was measured viscosimetrically (1), there is little or no inhibitory activity in the serum fractions to testicular hyaluronidase except in those containing albumin. Table II shows further that only fractions containing γ-globulin possess appreciable inhibitory action to streptococcal hyaluronidase. From the data it would appear that the purer the albumin the greater the antitesticular hyaluronidase activity. Thus, the fraction labeled albumin actually contained less albumin per ml. than did fractions labeled albumin plus α-globulin or albumin plus β-globulin, etc., as is schematically illustrated in Fig. 1, yet it possessed the highest activity. This statement does not hold, however, for the antistreptococcal activity of γ-globulin fractions, in which the inhibiting action seemed to be in proportion to the quantity of γ-globulin irrespective of the presence of other fractions. In experiments on the respective fractions.it was found that the inhibitor associated with albumin was heat-labile, being almost completely destroyed in 30 minutes at 56°, whereas the antistreptococcal hyaluronidase in γ-globulin was heat-stable, losing no activity under these conditions.

DISCUSSION

The differences in electrophoretic migration of the two inhibitors of hyaluronidase studied are so clear cut as to require no comment. The migration of the antistreptococcal inhibitor with the γ-globulin fraction is quite consistent with other evidence that this is a true antibody, such as the stability of this inhibitor to heating at 56° for 30 minutes mentioned above and the occurrence of this inhibitor in relation to streptococcal disease (4). On the other hand, it is likely that the inhibitor associated with albumin is not an antibody, because of its electrophoretic migration, its heat lability, and its disappearance from fresh serum after storage for more than a week in the refrigerator. It is more probably a substance which interferes with the action of the enzyme on its substrate. In this connection the failure of demonstration of the albumin-associate inhibitor when tested against the streptococcal enzyme is of some interest. In the sera thus far tested the concentration of this inhibitor was so low when tested against testicular enzyme that the negative results against streptococcal enzyme are of questionable significance. Should exploration of sera more rich in the albumin-associated inhibitor bear out the results obtained thus far, it would imply a difference between the two enzymes and their mode of action, such that one of the two is susceptible to interference by this inhibitor.

Of further interest is the fact that Fulton, Marcus, and Robinson (6) have reported an increase in thermolabile hyaluronidase inhibitor in the serum of humans with malignancies.

SUMMARY

The antihyaluronidase, which inhibits streptococcal hyaluronidase and which is found to be more concentrated in the sera of patients with streptococcal and rheumatic infection, migrates entirely with the γ-globulin of human sera and is thus distinct from the inhibitor of hyaluronidase described by Glick and Moore, which migrates with albumin.

Other characteristics of the two inhibitors indicate that the former is a true neutralizing antibody, and that the latter is not.

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STUDIES ON THE PHOTOCHEMISTRY OF 2-METHYL-1,4-NAPHTHOQUINONE*

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It has been known since the isolation of the vitamins K that derivatives of 2-methyl-1,4-naphthoquinone (MNQ) are unstable to light (1–5). However, the exact nature of the chemical change is still under discussion (6, 7). An interesting observation by MacCorquodale and coworkers (3) that the vitamin is quite stable as it exists in crude extracts, only becoming labile after a fair degree of purification, has apparently gone unnoticed. In subsequent investigations with the purified vitamin, elaborate precautions have been taken to avoid exposure to light.

In connection with an investigation in this laboratory of the metabolism and of the effect of x-ray irradiation[1] on the vitamins K, it was found that physiological sodium chloride solutions of MNQ are stable even when exposed to direct sunlight for periods of up to 1 hour. Consequently, an investigation of several substances was undertaken to determine which protect the vitamin in solution and what limits of concentration afford protection.

Ewing and coworkers (5) determined the rate of decomposition of vitamin K by ultraviolet light, and from the changes in the ultraviolet spectra thus produced, drew conclusions as to the nature of the chemical changes which took place. The results of similar experiments we have performed with MNQ in ultraviolet light and direct sunlight are illustrated in Figs. 1 and 2 respectively. Several points in these families of curves are of interest.

First, it may be noticed that, aside from the greater rate of change produced by sunlight compared with the Hanovia lamp, there appears to be a qualitative difference[2] in the curves which may point to some difference in the types of reactions involved and the products formed.

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* This report is based on work performed under contract No. AT-04-1-gen-12 with the Atomic Energy Commission for the University of California at Los Angeles.

[1] No change in the absorption spectrum of a 3.70×10^{-4} M solution of MNQ was observed after exposure to 330 roentgens per minute for 2 hours.

[2] For example, the curves in Fig. 2 do not pass through the isosbestic points of Fig. 1.

Second, as the group of maxima (Fig. 1) at about 250 mμ reduces in intensity under ultraviolet illumination a new maximum at 228 mμ[3] appears, increases somewhat in intensity, and then begins to decrease. This observation can most easily be explained as the result of consecutive reactions rather than as the formation of a single product from a single reaction, as postulated by other workers (6, 7). The nature of this change is being investigated further.

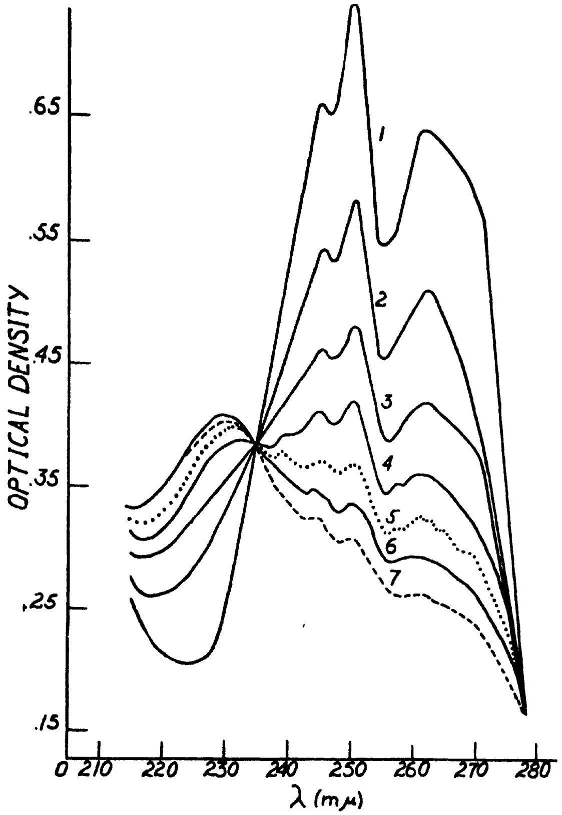

FIG. 1. Absorption curves showing the effect of ultraviolet radiation on 2-methyl-1,4-naphthoquinone in water. The readings for Curves 1 to 7 were taken at 0, 40, 80, 120, 160, 200, and 240 minutes respectively.

As Ewing noted for vitamin K_1, isosbestic points are evident at 278 and 235 mμ (Fig. 1). These points are a very convenient measure of the combined concentrations of MNQ and its decomposition products at any stage of the reaction. Finally, the freedom of MNQ from its decomposition products can be determined simply by a comparison of the intensity of the absorption at 228 mμ with that at 250 mμ.

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[3] Ewing *et al.* (5) apparently did not make observations in this region.

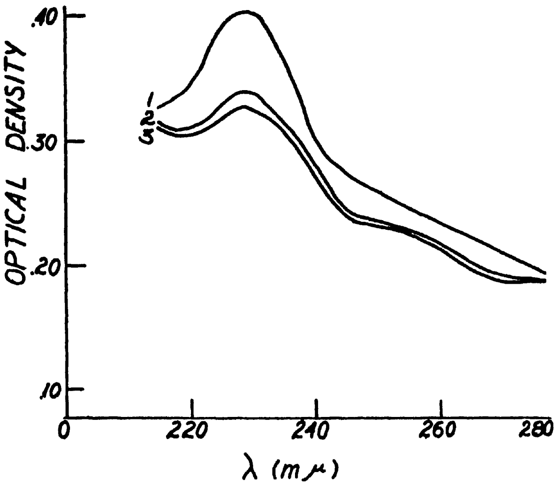

FIG. 2. Absorption curves showing the effect of sunlight on 2-methyl-1,4-naphthoquinone in water. The readings for Curves 1 to 3 were taken at 15, 60, and 120 minutes respectively.

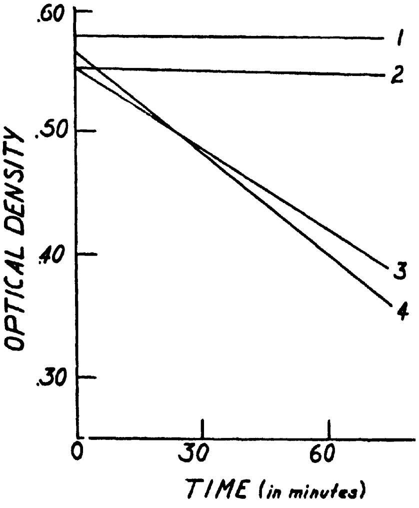

FIG. 3

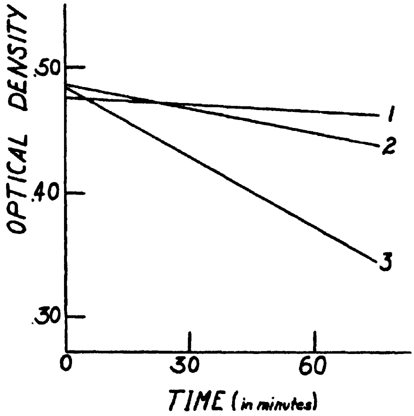

FIG. 4

FIG. 3. Effect of various anions on the decomposition of 2-methyl-1,4-naphthoquinone by ultraviolet light. The readings for Curves 1 to 4 were taken on KCl, NaBr, NaF, and H_2O solutions respectively.

FIG. 4. Effect of chloride ion concentration on the decomposition of 2-methyl-1,4-naphthoquinone by ultraviolet light. The readings for Curves 1 to 3 were taken on KCl solutions of 0.1, 0.01, and 0.001 per cent approximately, respectively.

In order to clarify the nature of the protective action of sodium chloride, the effect of sodium sulfate was determined. The observation that the latter did not protect solutions of MNQ against photochemical changes indicated that chloride ion was responsible for our initial finding and led us to test several other anions, with the results shown in Fig. 3. Bromide ion, no less than chloride ion, is seen to afford adequate protection. Other anions and substances not tried in this preliminary investigation may, of course, also have this effect.

Finally, the effect of chloride ion at several concentrations was determined to ascertain the lower limits affording protection. These results (Fig. 4) show that solutions of MNQ in 0.1 per cent KCl are quite stable. Even those in 0.01 per cent KCl are reasonably stable if the exposure to ultraviolet light is fairly short in duration.

Since chloride ion does not absorb light appreciably in the range concerned here, and since there is no interaction between chloride ion and MNQ (as indicated by the identity of the absorption spectra of solutions of the latter in the presence or absence of chloride ion), it seems fairly certain that the protection of MNQ against photochemical decomposition afforded by chloride, bromide, and (possibly) other ions is attributable to inactivation by these ions of the photon-activated MNQ molecule. Among numerous known instances of probably analogous phenomena, the most familiar is the quenching of the ultraviolet fluorescence of quinine solutions by chloride ion.

It is thought that this preliminary report may point the way to further investigations of the subject and also indicate a simple and inexpensive method of preventing the light-induced inactivation of vitamin K.

EXPERIMENTAL

Photochemical Decomposition of MNQ—2-Methyl-1,4-naphthoquinone (Eastman Kodak Company, White Label) was recrystallized[4] twice from absolute methanol, and 0.0289 gm. of the recrystallized product was dissolved in 20 ml. of 96 per cent redistilled ethanol and diluted with distilled water to 500 ml., giving a 3.34×10^{-4} M solution. This stock solution was stored in a volumetric flask and kept in a refrigerator until just prior to use, when it was allowed to warm to room temperature in the dark. The solution was stable throughout the entire period of research. 5 ml. of stock solution were diluted with distilled water to 50 ml., giving a dilution of convenient optical density. The absorption spectra of all solutions were taken on a Beckman spectrophotometer at wave-lengths from 215 to 280 mμ, with use of silica cells whose light paths were from 0.997 to 1.002 cm. The hydrogen lamp was used as a source of illumination.[5]

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[4] No change in absorption spectra was found after the first crystallization.

[5] It was ascertained that no destruction of MNQ occurred while the absorption cell was in the instrument.

The exposure of MNQ was carried out in an asbestos tube 69 × 15 × 15 cm., open at both ends, which was set up in a darkened fume hood. The source of illumination was a Hanovia ultraviolet lamp fitted with a No. 5032 filter. The wave-length of emitted light was 366 mμ.[6] No measure of the intensity of the radiation was made. The lamp was placed at the open end of the tube and masked in order to eliminate any stray radiation. Cooling of the lamp housing by several jets of compressed air was used to prevent heating the apparatus. The cells containing the solutions to be illuminated were placed in the Beckman cell holder and set in the tube, 30 cm. distant from the lamp.

After each exposure, the absorption spectrum was taken and the curves plotted.

Since this procedure was rather slow for large photochemical changes, exposure to direct sunlight was tried. It was observed that 15 minutes in direct afternoon sunlight caused a greater destruction of MNQ than 240 minutes exposure to the lamp. The curves obtained by scanning the spectrum from 215 to 278 mμ of a water solution of MNQ, which had been exposed to sunlight for periods of 15 minutes, 1 hour, and 2 hours, are shown in Fig. 2.

Protective Effects of Various Anions—5 ml. of 3.34×10^{-4} M MNQ solution were diluted to 50 ml. with 0.735 M sodium sulfate. Exposure of this solution to 15 minutes of direct afternoon sunlight showed no appreciable protection.

Solutions of 0.169 M KCl, NaBr, and NaF with MNQ were made up as above and exposed to the ultraviolet light of the Hanovia lamp, as previously described. The optical density of the solutions was taken at 250 mμ, giving the results illustrated in Fig. 3.

Concentration of Chloride Ion Necessary for Protection—To determine the concentration of KCl necessary to afford protection against destruction of MNQ by ultraviolet light, the 0.169 M KCl solution was diluted 10:1, 100:1, and 1000:1 to give approximately 1.69×10^{-2} M, 1.69×10^{-3} M, and 1.69×10^{-4} M concentrations of chloride ion. The decrease in the 250 mμ peak for various times of exposure to the Hanovia lamp source, as described above, is shown by the curves in Fig. 4.

SUMMARY

1. Existing knowledge of the chemistry of the light-induced decomposition of the vitamins K may be incomplete.
2. A simple method of protecting the vitamins from this decomposition

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[6] Illumination without the filter produced the same rate of change in the spectra of MNQ.

is proposed. The method consists of the addition of sodium chloride to solutions of the vitamins.

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A METHOD FOR THE DETERMINATION OF α-AMINO-β-HYDROXYISOVALERIC ACID (β-HYDROXYVALINE)*

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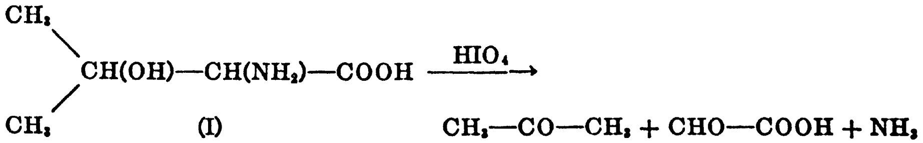
The possibility of the natural occurrence of α-amino-β-hydroxyisovaleric acid (β-hydroxyvaline) (I) has been indicated from several sources. The isolation of a "hydroxyvaline" was reported by Schryver and Buston (1), Brazier (2), and Czarnetzky and Schmidt (3), although these claims have been questioned (4, 5). Several workers have suggested the occurrence in proteins of hydroxyamino acids other than serine, threonine, and "hydroxylysine," but again the data are inconclusive (6–8). Also, the isolation of α-amino-β-thiolisovaleric acid (penicillamine) as a cleavage product of the penicillins (9) has suggested, by analogy with the known interrelationship of cysteine and serine, the possibility that the analogous hydroxy compound might occur. It seemed of interest, therefore, to devise a suitable method for the determination of β-hydroxyvaline in order to determine with some certainty the presence or absence of the compound in natural materials.

The procedure developed for the determination of β-hydroxyvaline is based on published methods (10, 11) for the determination of threonine and serine. The solution containing the free amino acids is treated with periodate. Under the conditions, serine yields formaldehyde, threonine yields acetaldehyde, and β-hydroxyvaline might be expected to yield acetone.


(I)

In the procedure for the determination of threonine and serine, the acetaldehyde is removed by aeration and collected in sodium bisulfite solution. In the presence of an excess of free amino acids, the formaldehyde is quantitatively retained in the original solution. The acetaldehyde may then be determined iodometrically. The formaldehyde is estimated by precipitation with dimethyldihydroresorcinol (dimedon) (11), or by colorimetric (12)

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TABLE I

Analysis for β-Hydroxyvaline in Some Natural Products

The figures in parentheses indicate the number of analyses performed.

Sample	β-Hydroxyvaline added before hydrolysis	β-Hydroxyvaline recovered*	Threonine found
	mg.	mg.	mg.
11.9 mg. threonine		13.0–13.4 (4)	11.7–11.9 (4)
10.0 " serine			
13.3 " β-hydroxyvaline			
200.0 " alanine			
2.0 " β-hydroxyvaline		2.0– 2.1 (4)	0.0 (4)
20.0 " alanine			
250.0 " casein		0.0 (4)	8.5– 8.6 (4)
250.0 " "	2.50	2.5 (2)	8.5– 8.6 (2)†
250.0 " "	0.50‡	0.5 (2)	8.6 (2)
200.0 " zein		0.0 (4)	6.1– 6.2 (4)
200.0 " "	2.00	2.0– 2.1 (4)	6.0– 6.2 (4)
200.0 " whole silk		0.0 (4)	7.6– 7.7 (4)
200.0 " " "	2.00	1.9– 2.1 (2)	7.6– 7.7 (2)
200.0 " *B. subtilis*		0.0 (2)	4.8 (2)
200.0 " *P. chrysogenum* mycelium, 20 hr.		0.0 (2)	1.8– 1.9 (2)
200.0 " " " " 20 "	2.00	2.1 (2)	1.8– 1.9 (2)
200.0 " " " " 40 "		0.0 (2)	1.8– 1.9 (2)
200.0 " " " " 60 "		0.0 (2)	1.8– 1.9 (2)
16.0 ml. *P. chrysogenum* culture filtrate, 0 hr.		0.0 (2)	8.0– 8.1 (2)
16.0 ml. *P. chrysogenum* culture filtrate, 20 hr.		0.0 (2)	7.0– 7.1 (2)
16.0 ml. *P. chrysogenum* culture filtrate, 20 hr.	2.00	2.0 (2)	7.2 (2)
16.0 ml. *P. chrysogenum* culture filtrate, 40 hr.		0.0 (2)	7.2 (2)
16.0 ml. *P. chrysogenum* culture filtrate, 60 hr.		0.0 (2)	7.0 (2)

* The recoveries are quantitative in every case, within the experimental error of the method (±5 per cent).

† Determinations of serine were run simultaneously by the method of Shinn and Nicolet (11) and yielded values of 11.3 to 11.7 mg. of serine. The sample analyzed was air-dried. If corrected for moisture (6.8 per cent) and expressed in the usual way as per cent of amino acid in the protein, the values for serine (4.9 per cent) and threonine (3.7 per cent) in casein compare reasonably well with published values (*cf.* (13, 20)). Apparently, the simultaneous determination of serine is feasible.

‡ In this experiment the β-hydroxyvaline was added after hydrolysis.

or iodometric (13) procedures. In experiments with β-hydroxyvaline, it was found that treatment with periodate yielded acetone. The acetone was removed from the reaction mixture by aeration and its identity established by preparation of the 2,4-dinitrophenylhydrazone (m.p. 123–124°; mixed melting point with authentic sample, 123–124°). However, under the conditions of the threonine procedure, the acetone was retained only partially but not quantitatively in the bisulfite solution. Available data on the reversible reaction of acetone with bisulfite (14) suggested that acetone might be quantitatively retained if the bisulfite solution was kept at a low temperature. This was found to be the case. Furthermore, it was established that, by more prolonged aeration, acetone could be completely removed from the bisulfite solution at room temperature while acetaldehyde was retained. Thus, the quantitative separation of formaldehyde, acetaldehyde, and acetone was possible and, therefore, the simultaneous determination of serine, threonine, and β-hydroxyvaline, if present in a single sample.

The procedure developed is described in detail in the experimental section. Results of recovery experiments on known mixtures of amino acids and of some analyses of natural products are included in Table I. By the method as little as 0.5 mg. of pure β-hydroxyvaline in 250 mg. of hydrolyzed protein can be estimated with some accuracy. Considerably smaller amounts could be detected. In no case were hydrolysates of the natural products found to contain a detectable amount of β-hydroxyvaline. Since β-hydroxyvaline added to the materials before hydrolysis was recovered in nearly quantitative yield in all cases, it seems probable that the compound does not occur in detectable amounts in these natural products. In the case of the purified proteins, the size of the samples (200 mg.) was such that one β-hydroxyvaline residue in a protein of molecular weight of 40,000 should have been readily estimated.

EXPERIMENTAL

*Preparation of β-Hydroxy-*DL*-valine*—β,β-Dimethylacrylic acid (15) was converted to α-bromo-β-methoxyisovaleric acid essentially by the procedure of West, Krummel, and Carter (16) for the preparation of the analogous α-bromo-β-methoxy-*n*-butyric acid.[1] β-Hydroxy-DL-valine was prepared from α-bromo-β-methoxyisovaleric acid by the method of Schrauth and Geller (17). The product melted at 217–219° (uncorrected); C 45.26, H 8.04, N 10.49; calculated for $C_5H_{11}O_3N$, C 45.11, H 8.26, N 10.52 per cent. The amino acid was converted to the *N*-benzoyl derivative by a customary procedure (18). The product was recrystallized from ethyl acetate and melted at 148.5–149.5° (uncorrected); C 60.61, H 6.31, N 5.92; calculated for $C_{12}H_{15}O_4N$, C 60.74, H 6.36, N 5.90 per cent; neutralization equivalent 237. Abderhalden and Heyns (5) report a melting point of 153° for the compound.

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[1] Dr. Vincent du Vigneaud and Dr. Herbert McKennis (private communication) have demonstrated this extension of the method.

Materials Analyzed

Casein—Vitamin test casein, control No. 1/16201, General Biochemicals, Inc., Chagrin Falls, Ohio.

Zein—Regular zein FH-54, supplied by Dr. A. L. Wilson, Corn Products Refining Company, Argo, Illinois.

Whole Silk—Obtained through the courtesy of Dr. Stanford Moore, The Rockefeller Institute for Medical Research, New York.

Bacillus subtilis (ATCC 6633)—A dried preparation of the organism harvested from submerged culture at the Western Regional Research Laboratory, United States Department of Agriculture, Albany, California, and supplied to Dr. James McGinnis of this institution.

Penicillium chrysogenum (Strain Q-176)—Representative samples of commercial batches from the production of penicillin were taken at different time intervals (0, 20, 40, and 60 hours) from the start of the fermentation. These were kindly supplied by Dr. K. S. Pilcher, Cutter Laboratories, Berkeley, California. The mold mycelium was separated by filtration, washed with water, and dried *in vacuo* at room temperature. Hydrolysates of the solid materials were prepared by heating the substance under a reflux with 12 volumes of 6 N hydrochloric acid. The excess acid was removed by repeated evaporation *in vacuo*. It was found necessary to treat the casein hydrolysate with a small amount of decolorizing carbon to prevent subsequent foaming during analysis. The hydrolysates of culture fluids were prepared similarly except that initially 7 ml. of concentrated hydrochloric acid were added to 5 ml. of culture filtrate. The total N content of the original culture fluids varied from 0.18 to 0.26 per cent.

Determination of β-Hydroxyvaline

Apparatus—The apparatus is similar to that of Shinn and Nicolet (10) except that two additional tubes are placed in the absorption train. This consists of five Pyrex test-tubes (2.5 × 20 cm.). The first tube is fitted with a dropping funnel which also serves as the gas inlet tube. It is convenient to carry out duplicate analyses simultaneously by arranging two trains in series, separated by a tube of saturated sodium bicarbonate solution.

Procedure—The five tubes are charged as follows: Into the first tube, which serves as the reaction tube, are introduced, in the following order, (1) the sample to be analyzed in a volume of 5 to 8 ml., (2) 5 ml. of 1 M sodium bicarbonate solution, and (3) 10 ml. of 0.1 N sodium arsenite solution, containing 20 gm. per liter of sodium bicarbonate. The second and third tubes contain 1 ml. of 2 per cent sodium bisulfite solution diluted to approximately 25 ml. with distilled water. The fourth and fifth tubes contain 10 ml. of 2 per cent sodium bisulfite solution diluted to approximately 25 ml. with distilled water. These last two tubes are immersed in an ice bath and allowed to reach the temperature of the ice bath before the reaction is started. The sodium bisulfite solution should be prepared fresh weekly and stored at 5°.

The apparatus is assembled and carbon dioxide is passed through for several seconds to mix the contents of the tubes. The gas inlet tube is then removed from the funnel and 2 ml. of 0.5 M periodic acid (H_5IO_6) are added with the stop-cock closed. The gas inlet tube is then connected and the periodic acid solution allowed to flow into the first tube. Carbon dioxide is passed through the system for 4 hours at the rate of about 1 liter per minute. During this entire period, the last two tubes are kept in an ice bath.

At the end of the aeration, the contents of the second and third tubes are titrated either separately or combined according to the following procedure: The excess bisulfite is titrated with the 0.05 N iodine solution (containing 8 gm. per liter of potassium iodide), with starch as indicator. 2 gm. of pulverized disodium hydrogen phosphate are added and the liberated bisulfite is titrated with 0.02 N iodine solution (prepared by dilution of the 0.05 N solution). The end-point is reached when the starch-iodine color persists for 30 seconds. 1 ml. of 0.02 N iodine solution is equivalent to 1.19 mg. of threonine.

The acetone collected in the fourth and fifth tubes is estimated iodometrically in a similar fashion, except that the titration with 0.05 N iodine solution is carried out at 5°. 2 gm. of disodium hydrogen phosphate are then added and the solution is placed in a water bath at 50° for 2 minutes. As suggested from previous studies (14, 19), it was found that quantitative results for acetone were not obtained if the sodium phosphate was replaced by sodium bicarbonate. 1 ml. of 0.02 N iodine solution is equivalent to 1.33 mg. of β-hydroxyvaline.

Although the amount of 0.05 N iodine solution used in the first titration need not be known, an excess of iodine at this point should be avoided. If the bisulfite originally added is measured carefully, the titration of the excess bisulfite may be used as an additional check on the amount of bisulfite bound.

SUMMARY

A procedure has been developed for the quantitative separation and estimation of small amounts of acetaldehyde and acetone. It has been

shown that oxidation of α-amino-β-hydroxyisovaleric acid (β-hydroxyvaline) with periodate yields acetone quantitatively. On the basis of these results, a method for the simultaneous determination of β-hydroxyvaline and threonine has been devised. No evidence was found for the occurrence of β-hydroxyvaline in any of the natural materials examined.

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BIOCHEMICAL STUDIES ON DIPHENHYDRAMINE (BENADRYL*)

I. CHEMICAL DETERMINATION OF DIPHENHYDRAMINE†

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The colorimetric determination of Benadryl (β-dimethylaminoethyl benzhydryl ether) is based on a general procedure for organic bases described by Brodie and Udenfriend (1). It depends on the reaction of the organic base with methyl orange to form a colored complex salt which is soluble in certain organic solvents. In using standard solutions of Benadryl in ethylene dichloride, it was found that methyl orange entered the organic phase in direct proportion to the concentration of Benadryl (2).[1] However, application of these earlier procedures to the analysis of urine and tissues gave high blanks, due to the presence of interfering substances. The method described here involves a double extraction technique which eliminates most of the interference, making it suitable for the determination of Benadryl in biological materials.

Reagents—

1. *Heptane.* Technical grade heptane is shaken for 5 minutes with one-fifth volume of 10 per cent NaOH. After separation, the hydrocarbon layer is washed successively with 1 N HCl and three separate portions of distilled water.

2. *Ethylene dichloride (EDC).* Technical grade EDC is purified by successive washes with alkali, acid, and water, as described for heptane.

3. *Borate buffer.* A 0.2 M borate buffer is made up by dissolving 12.4 gm. of boric acid and 14.9 gm. of KCl in about 800 ml. of water, adjusting the pH to 8.0 by the addition of 1 N NaOH, and bringing the final volume to 1000 ml.

4. *Methyl orange reagent.* 0.5 M boric acid solution is saturated with methyl orange by shaking overnight in a mechanical shaker. Any undissolved methyl orange is filtered and the solution is washed with three separate portions of EDC. The final reagent is stored in a bottle containing a lower layer of EDC.

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* Benadryl hydrochloride, registered trade name for diphenhydramine hydrochloride.

† These studies were reported in part at a meeting of the Federation of American Societies for Experimental Biology, March, 1948.

[1] Sultzaberger, J. A., unpublished data, Parke, Davis and Company (1945).

5. *Acid-alcohol reagent.* 2 ml. of concentrated sulfuric acid are mixed with 98 ml. of absolute ethyl alcohol.

Colorimetric Procedure

The final color is produced in EDC solutions containing up to 3.0 γ of free Benadryl base per ml. 0.5 ml. of the methyl orange reagent is added to approximately 10 ml. of the EDC solution in a glass-stoppered tube, and the mixture is shaken mechanically for 5 minutes. As much of the methyl orange layer as possible is aspirated, and the EDC layer is centrifuged at 1500 R.P.M. for about 10 minutes. The residual methyl orange collects at the sides of the tube.

5 ml. of the EDC layer are pipetted into 18 × 150 mm. test-tube cuvettes containing 0.5 ml. of the acid-alcohol reagent. Care is taken to see that no trace of the aqueous methyl orange reagent is transferred in this process. The contents of the cuvette are mixed thoroughly, and the optical density is read against a reagent blank by using a Coleman junior spectrophotometer set at 535 mμ.

The optical densities are referred to a standard curve prepared in the same manner, with known concentrations of Benadryl. Standard solutions of Benadryl hydrochloride are prepared in aqueous solutions, which are made alkaline and extracted into a known volume of EDC. Aliquots of the EDC are then taken for the colorimetric procedure. Typical results obtained in this manner on standard solutions of Benadryl are presented in Fig. 1.

EXPERIMENTAL

Extraction of Benadryl from Aqueous Solutions—In attempting to find the best organic solvent for extracting Benadryl from biological materials, a number of different systems were examined for distribution of the drug. It was found that EDC, heptane, and petroleum ether were satisfactory. The use of the less polar solvents (heptane and petroleum ether) produced lower blanks with normal biological samples than were obtained with EDC, indicating that smaller amounts of interfering substances were extracted. Heptane has proved to be more satisfactory than petroleum ether because it is easier to handle without appreciable evaporation losses.

Table I shows the effect of pH on the distribution of Benadryl between EDC and 0.2 M phosphate buffer at room temperature. The extraction of Benadryl was practically complete when the aqueous phase was on the alkaline side of pH 7.0. Similar results for heptane are presented in Table II, which shows that complete extraction of Benadryl is obtained when the pH of the aqueous solution is greater than 9.0. Shaking EDC or heptane with 0.1 N HCl resulted in complete transfer of the Benadryl to the acid layer.

Recovery of Benadryl from Plasma; Double Extraction Technique—The technique of double extraction is used to reduce the blank value of biological samples. This involves (*a*) extraction of Benadryl from an alkaline solution with heptane, (*b*) extraction of the heptane with dilute HCl, returning the Benadryl to the aqueous phase, and finally (*c*), after making the aqueous solution alkaline, reextracting the Benadryl into ethylene dichloride. The final color reaction with methyl orange is then performed directly on a portion of the EDC extract.

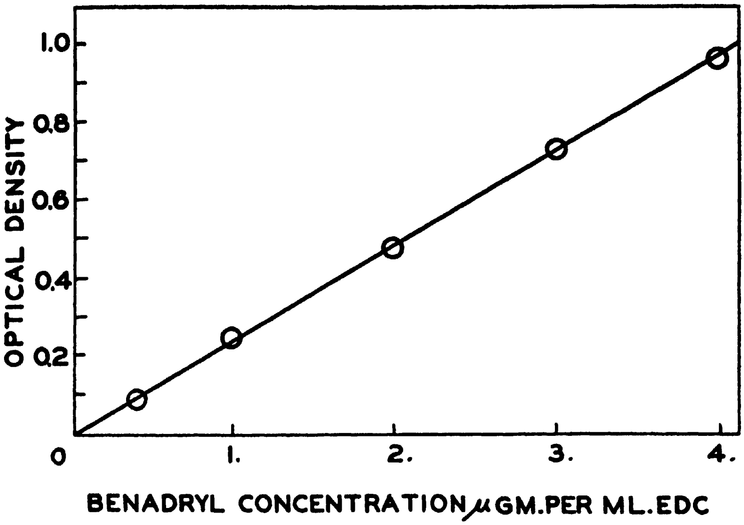

Fig. 1. Standard curve for Benadryl obtained by the methyl orange procedure. Readings were taken with a Coleman junior spectrophotometer at 535 mμ, with 18 × 150 mm. test-tube cuvettes.

Table I

Effect of pH on Extraction of Benadryl with Ethylene Dichloride

A mixture containing 1 ml. (25 γ) of Benadryl, 3 ml. of 0.2 M phosphate buffer, and 10 ml. of EDC was shaken for 10 minutes and centrifuged. The EDC layer was analyzed for Benadryl by the methyl orange procedure.

pH	Benadryl in EDC layer	Recovery
	γ	*per cent*
(0.1 N HCl)	0	0
4.0	5.7	23
5.0	20.4	82
6.0	24.4	98
7.0	24.8	99
8.0	25.6	102
(0.1 N NaOH)	25.0	100

The following procedure was used to establish recoveries of Benadryl from plasma: 1 ml. of standard solutions containing 0 to 30 γ of Benadryl was added to 3 ml. portions of oxalated human plasma in glass-stoppered bottles and made alkaline by the addition of 4 ml. of 0.1 N NaOH. The mixtures were shaken with 25 ml. of heptane for 10 minutes and then centrifuged. 20 ml. of the heptane phase[2] were then pipetted into clean bottles and shaken with 6 ml. of 0.1 N HCl for 5 minutes, the Benadryl being transferred to the acid layer. 5 ml. of the acid layer were pipetted into another tube, and to this were added 1 ml. of 1 N NaOH and 10 ml. of EDC. The mixture was shaken for 5 minutes, returning the Benadryl

TABLE II

Effect of pH on Extraction of Benadryl with Heptane

A mixture of 1 ml. (100 γ) of Benadryl, 10 ml. of 0.2 M phosphate buffer, and 11 ml. of heptane was shaken for 10 minutes and centrifuged. The heptane was aspirated and 1 ml. of 2.5 N NaOH added to a 3 ml. aliquot of the aqueous layer. This was extracted with 10 ml. of EDC, which was then analyzed for Benadryl by the methyl orange technique.

pH	Benadryl in heptane layer
	per cent total
(0.1 N HCl)	0
6.2	49
7.1	76
8.0	98
9.0	100
9.8	100
(0.1 N NaOH)	100

TABLE III

Recovery of Benadryl from Plasma

Benadryl added to plasma	Recovery
γ per ml.	*per cent*
0.5	93
1.0	97
2.0	100
5.0	104
10.0	102

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[2] With pure aqueous solutions of Benadryl in low concentrations, poor recoveries may be obtained due to adsorption on the glass containers. This can be overcome by the addition of a small amount of isoamyl alcohol to the heptane phase (3). However, we have experienced no such difficulties when biological materials containing Benadryl are extracted with heptane.

to the organic phase. The aqueous layer was then aspirated, and as much of the EDC as possible was transferred to a glass-stoppered tube for the methyl orange reaction. A blank determination was also made on the same plasma without any Benadryl added to correct for interfering substances. The results presented in Table III show good recoveries of Benadryl for concentrations between 1 and 10 γ per ml. of plasma.

The double extraction technique is also of value in reducing the possibility of Benadryl degradation products interfering with the determination. A single EDC extraction of an alkaline solution containing a small amount of β-dimethylaminoethanol was found to produce color in the methyl orange reaction, but no detectable amount was found in the EDC after the double extraction procedure via heptane and acid. Benzophenone and benzohydrol, other possible degradation products, gave no color by the methyl orange reaction.

TABLE IV

Recovery of Benadryl Added to Rat Tissues

20 γ of Benadryl were added to tissue homogenates, and per cent recovery was determined by the methyl orange technique after subtracting the blank value for normal tissue.

Tissue	Normal blank, Benadryl equivalents per gm. tissue	Benadryl recovered	Benadryl recovery
	γ	γ	*per cent*
Liver	0.1	20.2	101
Spleen	0.4	21.0	105
Heart	0.4	21.0	105
Muscle	0.1	20.7	103
Plasma	0.0	21.0	104
Red cells	0.1	19.2	96

Recovery of Benadryl from Tissues—Normal adult rats were sacrificed and different tissues removed for analysis. 1 gm. portions were weighed and homogenized in a motor-driven apparatus similar to the one described by Potter and Elvehjem (4), but with a stainless steel rotor in place of glass. Water was added during the homogenizing process,[3] and the final homogenates (about 5 ml.) were transferred to 60 ml. glass-stoppered bottles. 20 γ of Benadryl in aqueous solution were added to each sample. The solutions were made alkaline by the addition of 5 ml. of 0.1 N NaOH, and the mixtures were shaken with 25 ml. of heptane for 15 minutes. After separation, 20 ml. of the heptane layer were pipetted into another bottle and shaken with 6 ml. of 0.1 N HCl for 5 minutes. 1 ml. of 1 N NaOH was added to 5 ml. of the acid and shaken with 10 ml. of EDC. After separation by centrifuging, the EDC layer was taken for analysis by the methyl orange procedure. The same procedure was repeated without the addition of Benadryl to obtain normal tissue blanks, which are expressed as micrograms of Benadryl equivalents per gm. of tissue. The results are presented in Table IV. In all cases, the normal tissue blanks were quite low (0.1 to 0.4 γ per gm.) compared with the quantity of Benadryl present (20 γ per gm.).

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[3] Excessive dilution of the homogenate increased the tendency to form emulsions with heptane, whereas insufficient dilution made complete extraction difficult.

TABLE V

Distribution of Urinary Bases between Heptane and Aqueous Buffers

Heptane, containing organic bases extracted from normal urine, was shaken with an equal volume of 0.2 M phosphate or borate-KCl buffer and analyzed by the methyl orange technique after extraction into 0.1 N HCl and EDC. The results are expressed as per cent of original concentration of organic bases remaining in the heptane phase.

pH	Urinary bases remaining in heptane
	per cent
6.8 (0.2 M phosphate buffer)	6.3
7.0 (0.2 " " ")	7.8
7.2 (0.2 " " ")	11.8
7.4 (0.2 " " ")	16.5
7.6 (0.2 " " ")	20.4
7.8 (0.2 " borate-KCl ")	28.6
8.0 (0.2 " " ")	35.0
8.2 (0.2 " " ")	38.3
8.4 (0.2 " " ")	45.4

TABLE VI

Recovery of Benadryl from Urine by Various Extraction Procedures

Benadryl added per ml. urine	(a) EDC extraction		(b) Double extraction		(c) Double extraction + buffer wash	
	Optical density	Per cent recovery	Optical density	Per cent recovery	Optical density	Per cent recovery
γ						
0 (Blank)	0.124		0.013		0.005	
2.0	0.334	93	0.236	100	0.229	101
4.0	0.561	99	0.471	102	0.460	101

Procedure for Benadryl in Urine; Use of Alkaline Washes—Urine was found to give high blanks even with the double extraction technique because of the high concentration of other organic bases. This interference was reduced markedly by washing the initial heptane extract with an equal volume of a pH 8.0 borate-KCl buffer (see "Reagents").

In establishing the optimum conditions for washing the heptane extract, the distribution of normally occurring urinary bases between heptane and aqueous buffers was studied. Human urine was made alkaline and extracted with heptane to obtain the urinary bases which might produce interference. Aliquots of the heptane were then shaken with equal volumes of 0.2 M phosphate or borate buffers, and after separation of the two phases, the heptane was analyzed for organic base by the methyl orange technique, first extracting into 0.1 N HCl and then into EDC. The results are presented in Table V, expressed as per cent of the original concentration of urinary bases in the heptane phase. By using 0.2 M borate-KCl buffer at pH 8.0, it was found that 65 per cent of the urinary bases was removed from the heptane by a single extraction. Under the same conditions, less than 2 per cent of the Benadryl in the organic phase was removed.

To illustrate the effect of different extraction procedures on urinary blanks, normal human urine was extracted (*a*) directly into EDC, (*b*) by double extraction via heptane and acid into EDC, and (*c*) by double extraction in which the heptane phase was washed once with an equal volume of pH 8.0 borate buffer. Urine containing 2.0 and 4.0 γ of added Benadryl was also extracted in the same manner. Final EDC extracts representing equivalent volumes of urine were analyzed for organic base by the methyl orange technique. The results are presented in Table VI. The blank value found in procedure (*a*) was reduced 10-fold by the double extraction procedure (*b*), and 25-fold by the use of a buffer wash in procedure (*c*). Recoveries of Benadryl in procedure (*a*) were uncertain, due to the high blank, but in procedures (*b*) and (*c*) good recoveries were obtained with significantly lower urine blanks.

SUMMARY

Data are presented on various procedures for extraction of Benadryl from biological materials. A double extraction technique is recommended, involving extraction into heptane, transfer of the Benadryl back into acid solution, and from there into ethylene dichloride. In addition, an alkaline wash of the heptane extract is used where high concentrations of interfering substances are present. Final colorimetric measurements are made on the EDC solutions by the methyl orange technique for organic bases.

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BIOCHEMICAL STUDIES ON DIPHENHYDRAMINE (BENADRYL*)

II. DISTRIBUTION IN TISSUES AND URINARY EXCRETION†

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Benadryl has been used extensively as an antihistamine agent, but little is known concerning its mode of action or metabolic fate. It is rapidly absorbed, as shown by earlier blood level studies (1–3) and the fact that clinical effects are observed in less than an hour (4). This paper presents observations on the distribution of Benadryl in the rat, chosen for its resistance to the action of histamine, and in the guinea pig, which is well known for its sensitivity to histamine. In addition, evidence is presented for the presence of a small amount of unaltered Benadryl in urine.

EXPERIMENTAL

Distribution of Benadryl in Rat—Benadryl hydrochloride in aqueous solution was administered subcutaneously to a series of rats which had not been fed for 18 hours, with a dosage of 2 mg. per 100 gm. of weight. Groups of three rats were sacrificed at intervals of 1, 2, 4, and 6 hours after administration of the drug. The tissues were removed immediately after death and frozen at −20°. Analyses were usually completed within a few days. Tissues were homogenized and extracted with heptane, and the Benadryl was transferred by extraction to acid and finally to ethylene dichloride for colorimetric analysis (5). All results were corrected for normal tissue blanks. The three analyses for each time interval were averaged, with the results presented in Fig. 1. By far the highest concentration of Benadryl was found in lung tissue. Spleen was next highest, followed by kidney, brain, liver, and muscle. Peak concentrations were observed in 1 to 2 hours after administration of the drug.

Other routes of administration were studied, with the results shown in Table I. In this series of experiments, the rats were given 0.5 mg. of Benadryl hydrochloride per 100 gm. of weight by the routes indicated in Table I, and the animals were sacrificed 30 minutes after administration

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* Benadryl hydrochloride, registered trade name for diphenhydramine hydrochloride.

† Reported in part at a meeting of the Federation of American Societies for Experimental Biology, March, 1948.

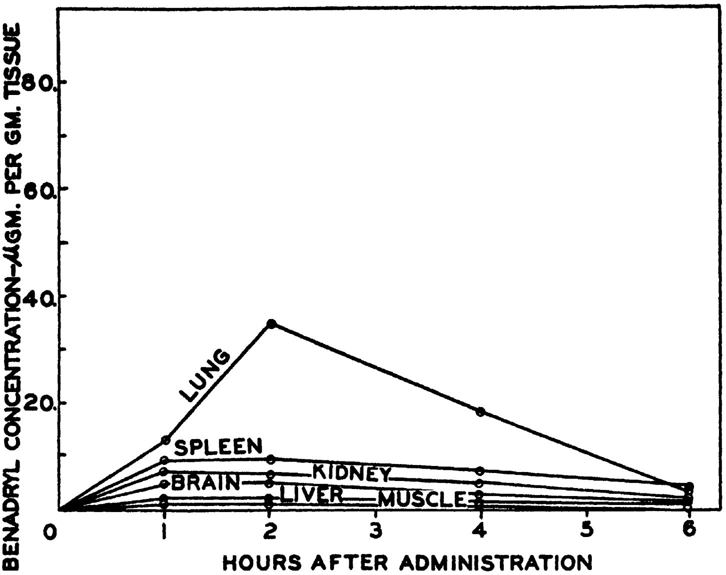

FIG. 1. Distribution of Benadryl in rat tissues. 2 mg. of Benadryl hydrochloride per 100 gm. of weight were administered subcutaneously. Each point represents the average results from three animals. Concentrations are expressed as micrograms of Benadryl base per gm. of tissue, corrected for normal tissue blanks.

TABLE I

Benadryl Concentration in Rat Tissues

0.5 mg. of Benadryl hydrochloride per 100 gm. of body weight was administered in aqueous solution by the routes indicated. The animals were sacrificed after 30 minutes, and the tissues analyzed for Benadryl. The figures express Benadryl concentrations as micrograms of free base per gm. of tissue.

Tissue	Route of administration			
	Oral	Intraperitoneal	Subcutaneous	Intravenous
Lung	0.6	4.6	5.7	13.7
Spleen	1.9	7.7	3.8	11.3
Brain	0.1	2.0	2.2	8.7
Liver	1.5	1.5	0.3	0.9
Muscle	0.1	0.5	0.5	2.3
Heart	0.6	1.3	1.5	3.5
Plasma	0.1	0.1	0.1	0.3
Red cells	0.8	0.3	0.3	

of the drug. The analyses again showed high concentrations of Benadryl in the lungs and spleen when Benadryl was administered parenterally. Oral administration resulted in elevated levels in the liver and spleen.

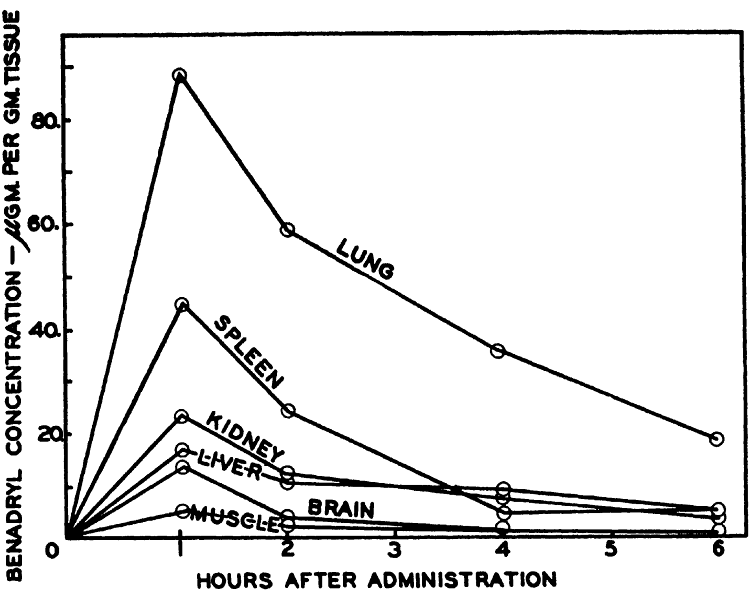

FIG. 2. Distribution of Benadryl in guinea pig tissues. 2 mg. of Benadryl hydrochloride per 100 gm. of weight were given subcutaneously. Each point represents the average results from three animals. Concentrations are expressed as micrograms of Benadryl per gm. of tissue, corrected for normal tissue blanks.

TABLE II

Benadryl Concentration in Guinea Pig Tissues

1 mg. of Benadryl hydrochloride per 100 gm. of body weight was administered in aqueous solution by the routes indicated. The animals were sacrificed 1 hour later, and tissues analyzed for Benadryl. The figures express Benadryl concentrations as micrograms of free base per gm. of tissue.

Tissue	Route of administration	
	Oral	Subcutaneous
Lung	14.9	76.6
Spleen	5.3	19.0
Brain	0.3	6.3
Liver	2.4	9.5
Muscle	0.4	3.7
Heart	0.6	6.7
Plasma	0.2	1.7
Erythrocytes	0.2	1.3
Skin		1.9

Distribution of Benadryl in Guinea Pig—The distribution of Benadryl was also investigated in the guinea pig. A series of animals (average weight 370 gm.) was injected subcutaneously with 2.0 mg. of Benadryl hydrochloride per 100 gm. of weight. The animals were sacrificed in groups of three at 1, 2, 4, and 6 hours after administration of the drug. Tissues were analyzed for Benadryl, as described earlier (5), with the results presented in Fig. 2.

It will be seen from this experiment that the order of tissue concentrations of Benadryl is similar to that observed in the rat. Although the same dosage of Benadryl per unit weight of animal was administered to the rat (Fig. 1) and guinea pig (Fig. 2), the latter showed from 2 to 3 times the concentration of Benadryl found in rat tissues.

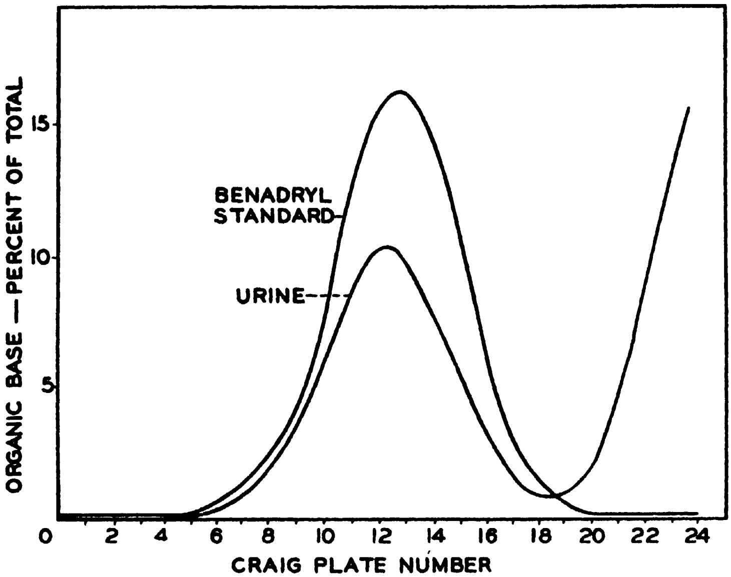

Fig. 3. Counter-current analysis of human urine for Benadryl. Urine extracts were run through the twenty-four plate Craig apparatus, with equal volumes of heptane and 0.135 M phosphate buffer at pH 6.13 for the counter-current extraction. The total organic base in each plate was determined by the methyl orange reaction. A pure sample of Benadryl hydrochloride was analyzed in the same manner.

The differences between the oral and subcutaneous routes of administration in the guinea pig were also compared. A series of guinea pigs was given 1.0 mg. of Benadryl hydrochloride per 100 gm. of weight by stomach tube or by subcutaneous injection, and the animals were sacrificed 1 hour later. Tissues were analyzed for Benadryl concentration, with the results shown in Table II. Here again, as in the rat, the concentration of Benadryl in lungs and spleen remains high, regardless of the route of administration.

Urinary Excretion of Benadryl—Numerous studies have been made on the urinary excretion of Benadryl, indicating that 5 to 15 per cent of the total dose is excreted (2, 3, 6) in 24 hours. Evidence is presented elsewhere (7) that non-basic degradation products are also present. The work described here demonstrates the presence of unaltered Benadryl in urine.[1]

The identity of Benadryl in urine was established by counter-current extraction with the Craig technique (8), and by the ultraviolet absorption spectrum of the purified material. 750 ml. of urine were obtained from normal human subjects given 50 mg. of Benadryl hydrochloride orally a few hours prior to urine collection. The urine was made alkaline by the addition of NaOH and extracted with an equal volume of heptane (5). The heptane was shaken with 50 ml. of 0.1 N HCl, which was then separated, made alkaline, and reextracted with 9 ml. of heptane. 8 ml. of this extract were fractionated in a 24 plate Craig counter-current apparatus, with equal volumes of heptane and 0.135 M phosphate buffer at pH 6.13. After the counter-current extraction, 1.5 ml. of N/3 HCl were added to each plate and the mixture shaken, the Benadryl being transferred completely to the aqueous phase. Aliquots of the aqueous phase were made alkaline and extracted into ethylene dichloride for colorimetric analysis by the methyl orange procedure (5). A counter-current extraction was also performed with a known sample of Benadryl hydrochloride under the same conditions. The results are presented in Fig. 3.

The close correspondence of the peaks in Fig. 3 is strong presumptive evidence that they are due to the same substance (8). When examined for ultraviolet absorption with a Beckman spectrophotometer, the samples in the middle of the series (Plates 9 through 14) had a peak absorption at 258 mμ, with a minor peak at 253 mμ, identical with that of a known sample of Benadryl.[2] The presence of other organic bases more water-soluble than Benadryl is demonstrated by the methyl orange color reaction towards the end of the series.

DISCUSSION

The high concentration of Benadryl in lung and spleen is of great interest because of the rôle played by these organs in anaphylactic shock. There is not sufficient evidence to say that Benadryl accumulates at "receptor sites" in competition with histamine, although that possibility should not be overlooked. However, analysis of guinea pig skin (Table II) gave low concentrations of Benadryl, even though the drug is known to affect cutaneous reactions attributable to histamine (9). Also, brain tissue, which normally has very little histamine present (10), showed fairly high concentrations of Benadryl on analysis (Figs. 1 and 2). The accumulation of Benadryl in various tissues apparently produces no pathological changes or blood dyscrasias (9), and toxic effects are promptly relieved by discontinuance of the drug, regardless of severity (11).

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[1] Since this work was completed, a paper by Hald (6) described the formation of a silicotungstic acid derivative of Benadryl from urinary sources.

[2] Spectroscopic examinations were made by Dr. J. M. Vandenbelt of the Research Laboratories, Parke, Davis and Company.

The concentration of Benadryl in the tissues of the rat and guinea pig showed significant differences (Figs. 1 and 2). In the rat, the level of Benadryl was one-half to one-third that shown by the guinea pig. This is perhaps better accounted for by the greater enzymatic activity of rat kidney and lung tissue than by differences in the rate of absorption or excretion of the drug (12).

We are especially indebted to Dr. A. C. Bratton, Dr. D. A. McGinty, and Dr. Graham Chen for their help and advice during the course of this work.

SUMMARY

Comparative data are presented on the levels of Benadryl in rat and guinea pig tissues at different time intervals following administration of the drug. After subcutaneous injection, the highest concentrations were found in the lungs, with progressively lower concentrations in the spleen, kidney, brain, liver, and muscle tissue. Peak concentrations were found in about 1 hour, with a fairly rapid drop towards normal levels in 6 hours.

The presence of a small amount of unaltered Benadryl was demonstrated in human urine by means of counter-current solvent extraction and ultraviolet absorption characteristics.

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BIOCHEMICAL STUDIES ON DIPHENHYDRAMINE (BENADRYL*)

III. APPLICATION OF RADIOACTIVE CARBON TO METABOLIC STUDIES OF BENADRYL

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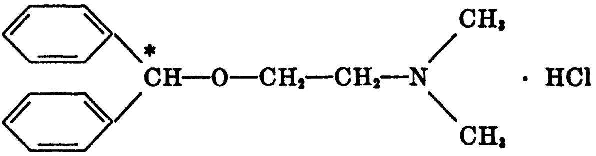
Biochemical studies on the urinary excretion of Benadryl have generally accounted for 5 to 15 per cent of the administered dose in 24 hours (1–3). Evidence has been obtained for the enzymatic degradation of Benadryl *in vitro*, the products no longer producing color in the analytical procedure for Benadryl (4, 5). The present work with a radioactive tracer demonstrates that metabolic products of Benadryl are formed in the body and excreted in the urine, thereby accounting for a greater percentage of the total dose than that found by chemical analysis.

Procedure

The experiments with radioactive Benadryl were performed with material synthesized by Dr. Robert Fleming and Dr. George Rieveschl (6). C^{14} was incorporated into the molecule in the α position of the benzhydryl group, as shown in the accompanying formula. The specific activity of this preparation was 224 μc. per gm., as estimated by the technique described below.



The asterisk indicates the position of C^{14} in the Benadryl molecule.

Urine and tissue extracts containing Benadryl or its degradation products were measured into porcelain micro combustion boats and evaporated to dryness. In all cases sufficient sample was taken to yield on combustion approximately 100 mg. of barium carbonate. Whenever this was impractical because of limited sample or low carbon content, sufficient benzoic acid was added to the sample to yield 100 mg. of $BaCO_3$. The samples were then placed in a micro combustion apparatus and burned in a stream of oxygen. The CO_2 was collected in 50 ml. centrifuge tubes containing CO_2-free 1 N NaOH, heated and precipitated as $BaCO_3$ by the addition of saturated $Ba(OH)_2$ solution. The $BaCO_3$ suspension was washed twice by centrifugation with CO_2-free water and then filtered by suction into a tared No. 1 Whatman filter paper (4.25 cm. in diameter), which was clamped with thin rubber gaskets between a Jena filter disk below and a cylindrical funnel of 1.9 cm., inside diameter, above. The walls of the funnel and the precipitate were washed first with water and then with 95 per cent alcohol in such a manner as to obtain fairly even distribution of the precipitate on the paper. After removal of the funnel, the filter paper and $BaCO_3$ disk were dried *in vacuo*, weighed, and then treated with a drop or two of 2 per cent ethyl acetate solution of isobutyl methacrylate polymer in order to reduce "dusting" of the $BaCO_3$ disk. The filter paper and $BaCO_3$ disk were then cemented to a 2 × 2 inch Dural slide, mounted uniformly under the Geiger-Müller tube, and radioactivity measured.

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* Benadryl hydrochloride, registered trade name for diphenhydramine hydrochloride.

Radioactivity counts were made with a Victoreen Geiger-Müller tube with a 2.7 mg. per sq. cm. mica window and a Tracerlab autoscaler. All radioactivities were corrected for resolving time, background, self-absorption, window and air absorption, and geometry and obliquity, according to methods described (7–9). The results are expressed as Benadryl hydrochloride equivalents, regardless of the chemical nature of the fraction measured.

The experiments on paper chromatography were performed by the methods described by Consden, Gordon, and Martin (10). A measured volume of urine (0.1 to 0.01 ml.) containing radioactive derivatives of Benadryl is evaporated in a narrow band 5 cm. from one end of a 1.5 × 60 cm. strip of filter paper. This is done by placing a piece of Nichrome resistance wire a few mm. below the area where concentration of the solutes is desired, and passing sufficient current through the wire to keep it hot. As the sample evaporates, water is fed in to either side of the heated area from water-saturated wicks of cloth. The rapid evaporation directly above the hot wire washes the solutes to that area, resulting in a very narrow band of solutes across the width of the strip. The paper is then dried in an oven at 100° and chromatographed as described elsewhere (10). After 16 hours, the solvent boundaries are marked and the paper strips are dried in an oven. The strips are then placed in close contact with Eastman No-screen x-ray film in a photographic printing frame and allowed to stand for 24 to 72 hours, after which time the film is developed. Dark bands appear on the film corresponding to areas of high radioactivity on the strips, and R_F values are calculated from the ratio of the distance traveled by the radioactive compounds to the distance traveled by the solvent from the top of the strip (10).

EXPERIMENTAL

Comparison of Chemical and Radioactivity Techniques for Assay of Benadryl in Tissues—Each of two guinea pigs was injected subcutaneously with 10 mg. of Benadryl hydrochloride per kilo of body weight. The animals were sacrificed 75 minutes after injection. The tissues were removed, frozen rapidly, and stored until analyzed. Corresponding tissues from the two animals were pooled, homogenized, make alkaline with NaOH, and extracted with petroleum ether as described in the first paper of this series (5). The petroleum ether layer was separated and extracted with a small volume of dilute HCl. One aliquot of this material was made alkaline and reextracted with ethylene dichloride for colorimetric analysis (5). A second aliquot was made alkaline and extracted with petroleum ether. This extract was prepared as a $BaCO_3$ disk for radioactivity measurement, as already described. Table I contains comparative data obtained by the two procedures, expressed as micrograms of Benadryl base per gm. of tissue.

TABLE I

Comparison of Benadryl Concentration in Guinea Pig Tissues by Chemical and Radioactivity Methods

Petroleum ether extracts of the tissue homogenates were analyzed for Benadryl by chemical and radioactivity assay methods.

Tissue	Concentration of Benadryl hydrochloride	
	Chemical method	Radioactivity measurements
	γ per gm. tissue	*γ per gm. tissue*
Lung	62.2	67.4
Spleen	17.8	17.9
Liver	9.6	7.8
Brain	5.1	5.7
Heart	2.4	4.3
Uterus	3.8	3.6
Blood	0.7	2.3
Skin	1.9	1.7
Muscle	1.3	1.6
Bile	0.0	0.0

It is evident from Table I that there is good correlation between the results obtained by the two methods. The order of concentration of the drug in the various tissues corresponds with the chemical assay values obtained in previously reported experiments (11). The results obtained indicate, furthermore, that the material extracted by petroleum ether from an alkaline solution consists of organic bases derived almost entirely from administered radioactive Benadryl. This fraction represents at least in part unchanged Benadryl itself, as was demonstrated by the Craig counter-current partition experiments (11).

Estimates of the total concentration of Benadryl in the body from the above data (75 minutes after injection) show that approximately one-third of the administered dose is accounted for in the major body tissues. The other two-thirds are probably in the form of degradation products which are not extracted under the conditions used for the separation of Benadryl.

TABLE II

Excretion of Benadryl and Radioactive Non-Benadryl Fractions Following Injection of Radioactive Benadryl Hydrochloride into Rats

Benadryl assay figures were obtained on petroleum ether extracts of the urine samples. The radioactivity of the residue following extraction was also measured.

Rat No.	Benadryl HCl, per 24 hrs.		Non-Benadryl residue γ Benadryl HCl equivalents per 24 hrs. (radioactivity)	Total Benadryl + non-Benadryl γ Benadryl HCl equivalents per 24 hrs. (radioactivity)	Total radioactivity of urine (per cent administered dose)	Non-Benadryl fraction (per cent total radioactivity of urine)
	Chemical assay	Radioactivity measurement				
	γ	γ				
1	298	303	1619	1922	38.5	84.2
2	227	180	1118	1298	26.0	86.1
3	296	273	1430	1703	34.1	84.0

Evidence for Presence of Benadryl Degradation Products in Urine—Each of three rats was injected subcutaneously with a solution containing 5 mg. of radioactive Benadryl hydrochloride. The rats were placed in metabolism cages and their urines collected for 24 hours. An aliquot of each urine collection was made alkaline with NaOH and extracted with a known volume of petroleum ether, an established procedure for extraction of Benadryl (5). Both the petroleum ether extract and the non-extracted residue were measured separately for radioactivity after combustion and preparation of $BaCO_3$ disks. The results of this experiment are shown in Table II.

Attention is again called to the fair agreement between the chemical and radioactivity assays for Benadryl. The most significant observation, however, is that, after extraction of organic bases from alkaline urine by petroleum ether, approximately 85 per cent of the total radioactivity remains in the aqueous phase in all three cases. This indicates that most of the Benadryl is excreted in a form which is not extracted by petroleum ether from alkaline solution. However, the total recovery of radioactive products represented only 26 to 38.5 per cent of administered radioactivity. The low excretion of Benadryl itself (4 to 6 per cent of administered Benadryl) is in agreement with previously published results (1–3).

The urinary excretion of Benadryl and its degradation products was also studied in the rat by parallel chemical and radioactivity assays on urine samples collected at different time intervals after administration of radioactive Benadryl hydrochloride. Three rats were injected subcutaneously with 2.0 mg. of radioactive Benadryl hydrochloride (aqueous solution) per 100 gm. of body weight. Urine was collected at 7, 23, 52, and 71 hours after injection, the metabolism cages and funnels being washed with distilled water to collect as much of the urine as possible. After measuring the volumes, part of each sample was assayed for total radioactivity by the barium carbonate technique. The rest was made alkaline and extracted with heptane, which was washed with pH 8.0 borate buffer, reextracted into HCl, and finally into ethylene dichloride for Benadryl assay by the methyl orange procedure (5).

TABLE III

Excretion of Benadryl and Degradation Products in Rats at Different Time Intervals Following Single Subcutaneous Dose of Radioactive Benadryl Hydrochloride (2.0 Mg. per 100 Gm. of Body Weight)

Chemical assay values for Benadryl and radioactivity measurements for whole urine are expressed as *per cent of administered dose* for each collection period. Radioactivity measurements on whole urine represent excreted Benadryl plus metabolic products of Benadryl.

Rat No.	Weight	Urine collection period							
		0 7 hrs.		7–23 hrs.		23–52 hrs.		52–71 hrs.	
		Chemical assay	Radioactivity measurement	Chemical assay	Radioactivity measurement	Chemical assay	Radioactivity measurement	Chemical assay	Radioactivity measurement
	gm.								
1	160	2.5	10.2	0.5	25.7	0	3.9	0	1.0
2	170	1.5	6.6	1.2	25.9	0	1.2	0	1.2
3	270	5.5	12.1	1.5	19.7	0	6.4	0	2.0
Mean		3.2	9.6	1.1	23.8	0	3.8	0	1.4

The results presented in Table III are calculated as per cent of original dose recovered in the urine. From these results, it can be seen that 33.4

per cent of the total administered radioactivity is recovered in 23 hours. In 71 hours, only 38.6 per cent of the administered dose is accounted for. About 11 per cent of the radioactive products excreted in this period responds to the methyl orange test for Benadryl. The average rate of excretion in micrograms of Benadryl hydrochloride equivalents per hour per 100 gm. of body weight is plotted in Fig. 1. The maximum rate of excretion of organic base (Benadryl) occurs in the first 7 hours, whereas the non-basic degradation products which are not detected by the methyl orange reaction are excreted over a much longer period of time, and represent the bulk of the excreted drug.

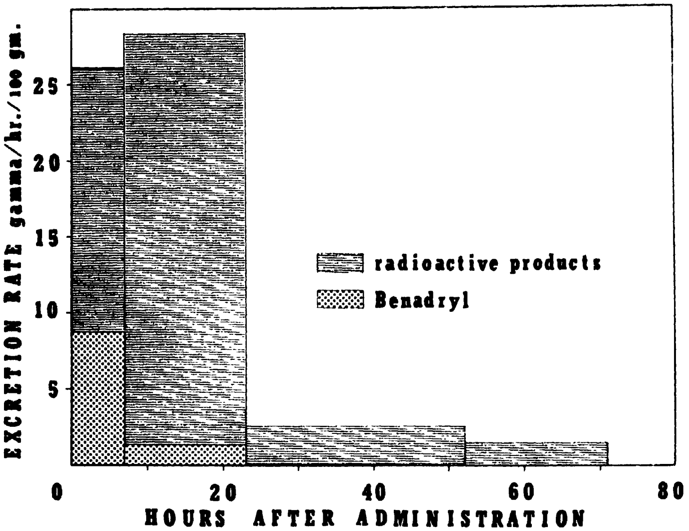

FIG. 1. Urinary excretion rate of free Benadryl and of radioactive metabolic products in the rat. Average excretion rate from three rats given 2 mg. of radioactive Benadryl hydrochloride subcutaneously per 100 gm. of body weight.

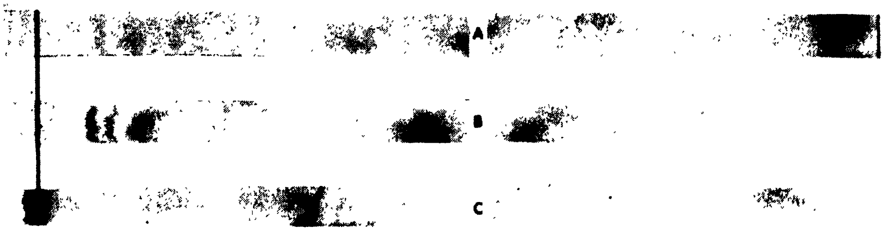

FIG. 2. Radioautographs from chromatographed urine of rats given radioactive Benadryl hydrochloride. The direction of the solvent travel is from left to right, with the right-hand mark indicating the limit of solvent travel. *A*, original urine chromatographed with *n*-butanol with 3 per cent NH_4OH added; *B*, residue after extraction of alkaline urine with ethylene dichloride; *C*, urine after acid hydrolysis.

Paper Partition Chromatography of Urine—Urine was collected from rats injected subcutaneously with radioactive Benadryl, and chromatographed on paper strips which were then radioautographed as already described. The results show that at least six different radioactive compounds are present in rat urine, all obviously containing C^{14} which was originally present in the Benadryl molecule. Normal rat urine chromatographed in the same manner showed no trace of the darkening on the photographic film, ruling out the possibility of chemical fogging of the emulsion.

The results of chromatography with *n*-butanol with 3 per cent ammonia added are shown in Fig. 2.

The original urine showed bands with calculated R_F values of 0.07, 0.12, 0.23, 0.37, 0.53, and 0.93 (Fig. 2, *A*). Unchanged Benadryl was shown to occur at $R_F = 0.93$, because radioactive Benadryl added to the urine produced a more intense band at this location. This observation is also supported by the identification of Benadryl in human urine, which was made earlier by use of the counter-current extraction procedure (11). In addition, extraction of the alkaline rat urine with ethylene dichloride resulted in complete removal of this band only (Fig. 2, *B*), leaving behind the other compounds which do not behave as organic bases. After acid hydrolysis of the urine in 1 N HCl for 1 hour at 100°, a single strong band appears at $R_F = 0.32$, with the elimination of all other bands (Fig. 2, *C*), except for a small amount of radioactive material remaining at the starting point. The R_F values appear to be influenced by the salt concentration of the urine, and vary to some extent in these experiments.

DISCUSSION

The determination of Benadryl in urine and tissues by chemical and radioactivity techniques shows fairly good agreement, indicating that the organic bases are in fact derived from Benadryl. That these bases represent unaltered Benadryl itself is borne out by the chromatography experiments described here, and by the Craig counter-current extraction experiments described earlier (11).

In addition to Benadryl, the chromatographic work shows that a number of other radioactive products are present in urine which do not behave as organic bases in their extraction properties. These are probably related in structure, since acid hydrolysis produces a derivative which chromatographs as a single band. The experiments on urinary excretion also show that the metabolic products of Benadryl are excreted in far greater concentration than unaltered Benadryl, and appear in the urine over a longer period of time.

SUMMARY

1. Benadryl analyses of tissues by chemical and radioactivity procedures show good agreement.

2. The urinary excretion of non-basic metabolic products of Benadryl is demonstrated by radioactivity assay, accounting for a larger percentage of the administered dose than heretofore possible.

3. The presence of Benadryl and a number of degradation products in rat urine is demonstrated by the preparation of radioautographs from paper strip chromatograms.

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BIOCHEMICAL STUDIES ON DIPHENHYDRAMINE (BENADRYL*)

IV. DEGRADATION OF BENADRYL BY TISSUE ENZYMES†

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The excretion of unchanged Benadryl accounts for only a small portion of the total administered dose in man (1, 2). Indirect evidence for the presence of degradation products in tissues and urine was obtained with Benadryl labeled with radioactive carbon (3). The experiments reported here show that degradation is produced by tissue enzymes, resulting in a loss of the basic properties of Benadryl as measured by the methyl orange technique (4).

EXPERIMENTAL

Comparative Activity of Various Tissues—Tissue samples were removed from the rat, guinea pig, and rabbit immediately after death. 1 gm. portions were weighed and minced thoroughly with a razor blade. The minced tissues were suspended in 5 ml. of Tyrode's solution at pH 7.4, containing 1:10,000 phenylmercuric lactate to inhibit bacterial growth. To this were added 50 γ of Benadryl hydrochloride in 1 ml. of saline. The mixtures were placed in an incubator room at 38° and shaken mechanically for 16 hours. The samples were then homogenized, extracted, and analyzed by the methyl orange procedure described in an earlier paper (4). The results, corrected for normal tissues blanks, are presented in Table I.

Of all the tissues tested, the liver showed greatest activity. Rat lung showed more activity than the corresponding tissue in the rabbit or guinea pig, and some activity was also evident in rat kidney. Heating the tissues to 60° for 10 minutes destroyed all activity. Benadryl incubated with the intestinal contents of a rat showed little or no loss of basic properties, indicating that the intestinal flora have no importance in this type of degradation of Benadryl.

Rate of Degradation—The rate of degradation of Benadryl was rapid enough to be detected in a few minutes under optimum conditions. 20 gm. of fresh rat liver were minced and suspended in 50 ml. of Tyrode's solution, and 1.2 mg. of Benadryl hydrochloride were added as an aqueous solution. The mixture was agitated mechanically in a 38° water bath, and 3 ml. samples were withdrawn periodically for analysis. The samples were heated to boiling immediately after withdrawal to stop enzymatic action. Samples were homogenized and analyzed for Benadryl by the methyl orange procedure (4). The results presented in Table II indicate that the rate of degradation of Benadryl by rat liver enzymes is proportional to the concentration of unchanged Benadryl, and resembles a first order reaction over the period covered by this experiment.

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* Benadryl hydrochloride, registered trade name for diphenhydramine hydrochloride.

† Reported in part at a meeting of the Federation of American Societies for Experimental Biology, March, 1948.

TABLE I

Degradation of Benadryl by Various Tissues

50 γ of Benadryl hydrochloride were incubated with 1 gm. of minced tissue in Tyrode's solution for 16 hours, and then analyzed for Benadryl. The amount of degradation is expressed as per cent of Benadryl originally added.

Tissue	Rat	Guinea pig	Rabbit
Liver	97	96	99
Lung	70	9	14
Kidney	25	8	27
Brain	6	8	4
Heart	6	5	5
Spleen	4	2	1
Muscle	3	4	13

TABLE II

Rate of Degradation of Benadryl

Benadryl hydrochloride was incubated with minced rat liver at 38° and samples were withdrawn at stated intervals for analysis by the methyl orange procedure.

Time (t)	Unchanged Benadryl (c)	$K = \left[\frac{\log (100/c) - 0.285}{t}\right]$
min.	*per cent original*	
0	100	
15	48.2	0.0021
30	44.7	0.0022
45	39.5	0.0026
60	37.1	0.0024
90	32.1	0.0023
120	24.2	0.0028

Effect of pH on Benadryl Degradation—0.9 gm. portions of rat liver were weighed and minced thoroughly with a razor blade. The minced tissue was transferred immediately to flasks containing 3 ml. of Tyrode's solution, 3 ml. of borate, phosphate, or acetate buffer, 0.5 ml. (50 γ) of Benadryl hydrochloride standard, and 0.5 ml. of a 1:1000 phenylmercuric lactate solution. The buffers were adjusted previously to different pH values by the addition of acid or base. After addition of the minced tissue, the flasks were incubated in a 38° water bath for 2 hours, a shaking device being used to keep the mixtures agitated. At the end of the incubation period, the pH of each mixture was measured with a Beckman pH meter and a high alkali glass electrode. The solutions were then heated in a boiling water bath for a few minutes to destroy enzyme activity. Control tests showed that Benadryl was not destroyed by heating under these conditions. The contents of each flask were then homogenized, made alkaline, extracted with heptane, and analyzed for Benadryl by the double extraction technique (4). The results are presented in Table III.

Table III

Effect of pH on Benadryl Degradation

Solutions of Benadryl in various buffers were incubated with minced rat liver at 38° for 2 hours. Acetate and phosphate buffers were used with the liver of one rat; a second rat was used for the borate experiment. Final pH readings were taken at the end of the incubation period. The results are calculated from the decrease in benadryl concentration as determined by the methyl orange procedure.

Buffer	Final pH	Benadryl degradation
		per cent
0.1 M acetate	3.9	0
0.1 " "	4.4	0
0.1 " "	5.3	11
0.067 M phosphate	6.1	29
0.067 " "	6.8	54
0.067 " "	7.4	66
0.1 M borate	6.9	69
0.1 " "	7.3	88
0.1 " "	8.3	51
0.1 " "	8.8	38
0.1 " "	8.9	24

The optimum pH for the enzymatic degradation of Benadryl appears to be in the physiologic range. Inasmuch as different liver preparations and different buffers were used in these experiments, no attempt was made to define the optimum pH with greater accuracy.

Effect of Enzyme Inhibitors—3 gm. portions of minced rat livers were suspended in 5 ml. of Tyrode's solution, and 1 ml. of various enzyme inhibitors was added. When necessary, the pH was readjusted to 7.4. Gases were introduced by bubbling through the mixture for several minutes, and the flasks were tightly stoppered. After addition of the inhibitor, 1 ml. of a solution of Benadryl hydrochloride (50 γ) was added to each flask. The mixtures were incubated at 38° for 2 hours with mechanical agitation. After homogenizing and extracting with heptane, Benadryl was determined by the methyl orange procedure (4). The results are presented in Table IV.

Very marked inhibition was produced by reducing agents such as cysteine, hydroxyamine, and hydrogen sulfide. Iodoacetate and azide showed strong inhibition, but cyanide and carbon monoxide had little effect. The greatest degree of inhibition, however, was obtained in a nitrogen atmosphere. Displacement of the air by nitrogen resulted in complete cessation of activity, as measured by quantitative recovery of Benadryl. Incorporation of hydrogen acceptors such as methylene blue had no effect on this inhibition. Similar results were obtained with other inert gases. Molecular oxygen therefore appears to be essential for the enzymatic degradation of Benadryl.

TABLE IV

Effect of Inhibitors on Degradation of Benadryl

Benadryl hydrochloride was incubated with minced rat liver in Tyrode's solution with various inhibitors added. At the end of the incubation period, samples were heated to stop enzyme action and analyzed for Benadryl by the methyl orange procedure.

Inhibitor	Unchanged Benadryl
	per cent original
Cysteine (0.050 M)	90
Hydrogen sulfide	73
Hydroxylamine (0.050 M)	59
Iodoacetic acid (0.001 M)	60
Sodium azide (0.002 M)	50
Carbon monoxide	1–20
Hydrogen peroxide (0.050 M)	7
Sodium cyanide (0.002 M)	4–8
" fluoride (0.0024 M)	0

DISCUSSION

Liver appears to be the principal site of Benadryl degradation, although rat lung and kidney are also active. No attempt was made to establish the precise activity of each tissue, but certain species differences are indicated in Table I. The lower activity of guinea pig lung and spleen may account for the higher concentrations of Benadryl found in guinea pig tissue (5).

The disappearance of the methyl orange color reaction is strongly indicative of the loss of basic properties. Such a condition might occur through rupture of the ether linkage, yielding dimethylaminoethanol and benzohydrol. Neither of these compounds gives any color with methyl orange, following double extraction into ethylene dichloride via heptane and 0.1 N HCl, although Benadryl is extracted quantitatively by this procedure (4). Way *et al.* (2) have presented evidence for the presence of some degradation products which react with methyl orange, but our results indicate that the major degradation products do not interfere with the determination of Benadryl by the double extraction procedure.

SUMMARY

The basic properties of Benadryl are lost through the action of a tissue enzyme system which is partly characterized. The liver appears to be the best source of this enzyme, with some activity observed in lung and kidney tissue. The reaction appears to be monomolecular, with the optimum pH in the physiologic range. Oxygen is required for the reaction, and various reducing agents are shown to act as inhibitors.

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COMPETITIVE ANTAGONISTS OF THYROXINE AND STRUCTURALLY RELATED COMPOUNDS*

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Woolley (1) has prepared a number of ethers of *N*-acetyl-3,5-diiodotyrosine and found them to interfere with various effects of thyroxine on amphibian larvae. Some of Woolley's observations were confirmed and extended by Winzler and Frieden (2) and by Williams *et al.* (3).

It is the purpose of this report to investigate the competitive nature of several thyroxine inhibitors and to determine the structural requirements for and the specificity of the inhibition of thyroxine and its active analogues.

Materials

*O-Benzyl-3,5-diiodo-*DL*-tyrosine* (*I*)—10 gm. of *O*-benzyl-*N*-acetyl-3,5-diiodo-L-tyrosine, m.p. 87–90°,[1] prepared as described by Woolley (1), were refluxed overnight in 1 liter of 1 N NaOH. The solid which precipitated at pH 5 was extracted with hot 0.2 N HCl, which when cooled yielded the crystalline hydrochloride of I. Free I, m.p. 203–205° with decomposition, was obtained in 30 per cent yield by dissolving its hydrochloride in dilute alkali and adjusting the pH to 5. The product gave a positive ninhydrin, but a negative Kendall, test for orthodiiodophenols. The above recrystallization process was repeated on a sample used for elementary analysis.

$C_{16}H_{15}O_3NI_2$. Calculated, C 36.7, H 2.9; found, C 36.9, H 3.0

4-Benzyloxy-3,5-diiodobenzoic Acid (*II*)—3,5-Diiodo-4-hydroxybenzoic acid, m.p. 275–277°, was prepared in 50 per cent of the theoretical yield from 4-hydroxybenzoic acid (Eastman Kodak, m.p. 214.5–215.5°) (4). 10 gm. of the iodinated phenol were dissolved in 60 ml. of 1 N NaOH, heated on a boiling water bath, and treated dropwise with 11.9 gm. of benzyl chloride. A heavy white precipitate of the sodium salt of II was formed on complete cooling, after the removal of a tarry by-product on preliminary cooling. II was generated by acidifying a suspension of the isolated salt. Two recrystallizations from a water-methanol mixture resulted in a 20 per cent yield[2] of II, m.p. 227–228°, which gave a negative Kendall test.

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† Some of the data were taken from a thesis presented by Earl Frieden to the Graduate School of the University of Southern California in partial fulfilment for the requirements for the degree of Doctor of Philosophy.

[1] All melting points are corrected.

$C_{14}H_{10}O_3I_2$. Calculated, C 35.0, I 52.9; found, C 35.2, I 52.9

3,5-Diiodo-4-(p-nitrophenylethoxy)-benzoic Acid—10 gm. of 3,5-diiodo-4-hydroxybenzoic acid were dissolved in 85 ml. of 0.7 N methanolic NaOH and treated with 6.1 gm. of *p*-nitrophenylethyl bromide, m.p. 64°, prepared as described by Woolley (1). The mixture was refluxed for 2 hours and concentrated to dryness under a vacuum, and the residue was thoroughly washed with 200 ml. each of warm water and ethyl ether. The washed residue was suspended in 100 ml. of water, the pH adjusted to 2.5 ± 0.5 with HCl, and the mixture heated for 2 hours. The resulting washed solid was twice recrystallized from an ethanol-water mixture, giving a 20 per cent yield of the ether, m.p. 219–220°, which gave a negative Kendall test.

$C_{15}H_{11}O_5NI_2$. Calculated, C 33.4, N 2.6; found, C 33.4, N 2.5

3,5-Diiodo-4-(4′-methoxyphenoxy)-aniline (III)—The preparation of III from 3,5-diiodo-4-(4′-methoxyphenoxy)-nitrobenzene was accomplished by adapting a procedure of Block and Powell (5) instead of the earlier method of Harington and Barger (6). 10 gm. of the nitro compound were suspended in 200 ml. of a 50 per cent ethanol solution containing 10 ml. of glacial acetic acid. 5 gm. each of 30 mesh iron filings and iron powder were added, and the mixture refluxed for 3 hours. The alcohol was removed by distillation, and the residue was cooled and repeatedly extracted with a total of 300 ml. of boiling benzene. Concentration of the benzene solution to about 150 ml. and the addition of 80 ml. of petroleum ether gave, with scratching, 75 to 80 per cent yield of III, m.p. 121–122°.

3,5-Diiodo-4-(4′-hydroxyphenoxy)-aniline (IV)—5 gm. of III were refluxed for 2 hours in 40 ml. of glacial acetic acid and 50 ml. of 42 per cent HBr. After diluting with 1 volume of water and cooling overnight, a brownish impurity was removed by filtration. Further dilution of the filtrate with 1 additional volume of water gave a 60 per cent yield of the hydrobromide of IV. Pure IV, m.p. 221.5–223.5°, was obtained by crystallization of its neutralized salt from 95 per cent ethanol.

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[2] A 60 per cent yield of II has been obtained subsequently by treating the dipotassium salt of 3,5-diiodo-4-hydroxybenzoic acid with 6 equivalents of benzyl chloride in hot 50 per cent ethanol and hydrolyzing the resulting benzyl ester of II with dilute NaOH.

$C_{12}H_9O_2NI_2$. Calculated, C 31.8, N 3.1; found, C 32.0, N 3.0

3,5-Diiodo-4-anisic acid was prepared as described by Wheeler and Liddle (7), m.p. 257–258°, compared to the reported melting point of 255–256°. 4-Benzyloxybenzoic acid was synthesized by essentially the same procedure described for the corresponding 3,5-diiodo derivative. A 30 per cent yield of crude product melting several degrees below the reported value of 188–190° was obtained.

N-Acetyl-DL-thyroxine, 3,5-diiodo-4-(4′-hydroxyphenoxy)-benzoic acid, 3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid, *N*-acetyl-3,5-diiodo-L-tyrosine, and the glycine homologue of thyroxine were obtained as described in a previous report (8). 2-Thiouracil and 2-mercaptoimidazole were kindly contributed by Eli Lilly and Company, Indianapolis, Indiana. 3,5-Difluoro-4-methoxyphenol and 3,5-dichloro-4-anisic acid were obtained through the generosity of Dr. J. F. Mead, Atomic Energy Project, University of California at Los Angeles. DL-Thyroxine was provided by Dr. K. W. Thompson of Roche-Organon, Inc., Nutley 10, New Jersey. All the other compounds tested were the best available grade obtainable from the Eastman Kodak Company.

Methods

The effect of thyroxine and compounds possessing thyroxine-like activity on amphibian metamorphosis afforded a convenient system for the study of compounds for thyroxine antagonism. The technique employed in these studies was similar to that described in previous work on the thyroxine-like activity of compounds structurally related to thyroxine (8). Duplicate dishes containing a solution of the active compound and the inhibitor were brought to a final pH of 8.0 ± 0.5 prior to introducing the tadpoles. In so far as possible, tadpoles of the same history, of the same relative nutritional state, and of the same length or pooled groups of length varying no more than 2 mm. were employed.

Results

Competitive Inhibition of Thyroxine—The results shown in Tables I to III summarize several of many experiments and indicate a competitive antagonism between thyroxine and a number of related compounds.

In Table I the action of thyroxine in inducing the metamorphosis of tadpoles is observed to be inhibited by *O*-benzyl-*N*-acetyl-3,5-diiodo-L-tyrosine, a compound reported by Woolley (1) to antagonize the toxicity of thyroxine to tadpoles. The thyroxine effect is reduced by 50 per cent when the molar ratio of inhibitor to thyroxine is about 550, regardless of their absolute concentrations.

Table II shows that *O*-benzyl-3,5-diiodo-DL-tyrosine is some 5 times more effective a thyroxine antagonist than its *N*-acetyl derivative, the molar ratio of inhibitor to thyroxine necessary to reduce the thyroxine effect by one-half being about 37. This is in accord with previous observations (8, 9) that N acetylation reduced thyroxine-like activity.

TABLE I

Competitive Inhibition of Thyroxine by O-Benzyl-N-acetyl-3,5-diiodo-L-tyrosine

O-Benzyl-*N*-acetyl-3,5-diiodo-L-tyrosine	Per cent decrease in length after 72 hrs. incubation				
	DL-Thyroxine per ml.*				
	0.25 γ	0.50 γ	0.75 γ	1.00 γ	1.25 γ
γ per ml.					
0	26	47	60	60	60
62.5	19	45	55	56	55
125	13	37	45	47	50
250	11	16	25	35	43
375	6	13	20	30	35
500		12	19	25	29
750		8	17	17	25
$(I/T)_{50\%}$ weight†	500	400	300	375	400
$(I/T)_{50\%}$ molar‡	688	550	412	515	550§

* No detectable difference in length from untreated controls was observed during the reported incubation period at all the indicated concentrations of the inhibitor in the absence of thyroxine.

† Ratio by weight of inhibitor to thyroxine required for reduction of thyroxine effect by 50 per cent.

‡ Molar ratio of inhibitor to thyroxine required for reduction of thyroxine effect by 50 per cent.

§ This value of (I/T) at highest inhibitor concentration is used in subsequent discussions because it is least affected by unsaturation of the system with respect to inhibitor or thyroxine.

The observation of gradually decreasing ratios with increasing thyroxine concentrations shown in Table II is in agreement with calculations made from the competitive inhibition equation of Lineweaver and Burk (10) for the relatively non-saturating ranges of concentrations of inhibitor and thyroxine used in the experiments reported in this paper. Further verification of the competitive nature of this type of antagonism appears in Fig. 1, *A* and *B*. A direct plot of per cent decrease in length against thyroxine concentration gives with increasing inhibitor a series of curves with a progressively decreasing effect at the same level of thyroxine (Fig. 1, *A*).

TABLE II

*Competitive Inhibition of Thyroxine by O-Benzyl-3,5-diiodo-*DL*-tyrosine*

O-Benzyl-3, 5-diiodo-DL-tyrosine	Per cent decrease in length after 63 hrs. incubation			
	DL-Thyroxine per ml.			
	0.25 γ	0.50 γ	1.00 γ	2.00 γ
γ per ml.				
0	28	46*	51*	60*
1.25	28	44*	48*	56*
2.50	24	44*	46*	56*
5.00	20	40	44*	50*
12.5	15	25	42*	44*
25.0	10	16	28	34
50.0	7	9	15	28
125	4	6	13	24
250	3	3	9	12
$(I/T)_{50\%}$ weight	50	30	30	25
$(I/T)_{50\%}$ molar	74	45	45	37

See corresponding foot-notes to Table I.

* Indicates 80 to 100 per cent died several hours prior to observation time. The protective action of this inhibitor against higher concentrations of thyroxine is thus clearly established.

TABLE III

Competitive Inhibition of Thyroxine by 4-Benzyloxy-3,5-diiodobenzoic Acid

4-Benzyloxy-3, 5-diiodobenzoic acid	Per cent decrease in length after 69 hrs. incubation				
	DL-Thyroxine per ml.				
	0.15 γ	0.30 γ	0.50 γ	0.90 γ	1.80 γ
γ per ml.					
0	19	30	45	48	55
0.50	17	23	35	40	46
1.00	8	19	30	32	43
2.50	4	8	20	25	40
5.00	2	4	18	20	37
7.50	2	4	10	12	33
10.0	*	*	*	10	25
$(I/T)_{50\%}$ weight	6	5	4	3	5
$(I/T)_{50\%}$ molar	9.5	8	6.5	5	8

See the corresponding foot-notes to Table I.

* Indicates toxicity of inhibitor dose.

A graph of $100/E$ against the reciprocal of the molar concentration of DL-thyroxine as shown in Fig. 1, *B*, where *E* is the per cent decrease in length at constant time, yields a family of lines with a common intercept and slope increasing with increasing amounts of inhibitor, characteristic of competitive inhibition (10). Non-competitive inhibition would give lines of variable intercept as well as variable slope.

The recent observation (8) that the carboxylic acid analogue[3] of thyroxine has a thyroxine-like activity of about 10 per cent of that of thyroxine suggested the investigation of ethers of 3,5-diiodo-4-hydroxybenzoic acid. The benzyl ether proved to be the most potent competitive inhibitor of all, the data in Table III indicating that molar ratios of inhibitor to thyroxine of 8 produced a 50 per cent reduction in thyroxine effect. The toxicity of this compound prevented its use at concentrations above those listed. The methyl and *p*-nitrophenylethyl ethers of 3,5-diiodo-4-hydroxybenzoic acid required molar ratios of 150 and 75, respectively, to produce the same antagonism. The importance of the 3,5-iodines was indicated by the failure of 4-anisic acid and 4-benzyloxybenzoic acid to inhibit appreciably at molar ratios of 500 and 350, respectively.

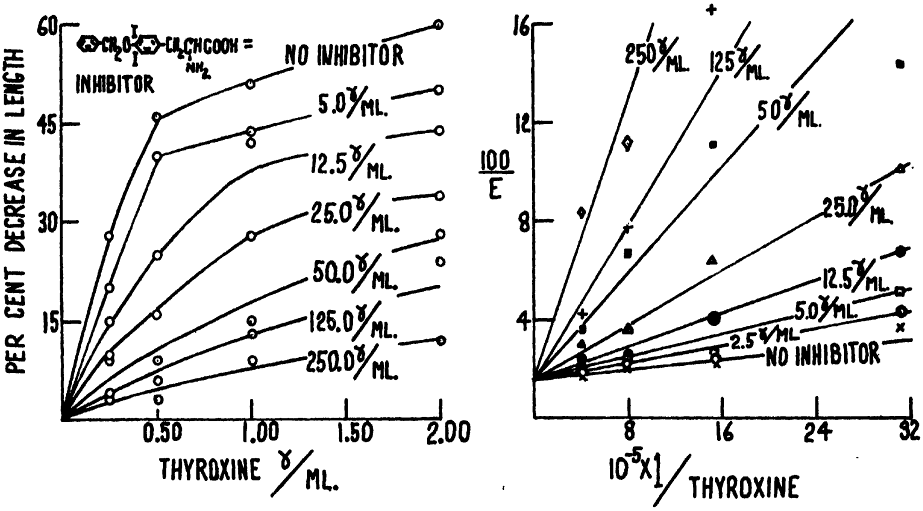

Fig. 1, *A*
Fig. 1, *B*

Fig. 1, *A*. The effect of increasing concentrations of *O*-benzyl-3,5-diiodo-DL-tyrosine on the response of tadpoles to DL-thyroxine concentration.

Fig. 1, *B*. The reciprocal of the observed effect as measured by the per cent decrease in the length of tadpoles plotted as a function of the reciprocal of the molar concentration of DL-thyroxine for various levels of *O*-benzyl-3,5-diiodo-DL-tyrosine.

Inhibition of Compounds with Thyroxine-Like Activity—The inhibition by thyroxine antagonists of the thyroxine-like activity of compounds with altered acid side chains was also observed. The action of the carboxylic acid analogue of thyroxine, 3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid, was competitively reduced by thyroxine antagonists at molar ratios similar to those for thyroxine. This is illustrated in Table IV which shows that the antagonism by *O*-benzyl-3,5-diiodo-DL-tyrosine of the thyroxine-like activity of the carboxylic acid analogue requires a molar ratio of 36 to diminish the thyroxine effect by 50 per cent. Ratios of 5 and more than 400, respectively, were required for a similar effect on the activity of this compound by 4-benzyloxy-3,5-diiodobenzoic acid and *O*-benzyl-*N*-acetyl-3,5-diiodo-L-tyrosine.

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[3] This designation has been preferred to the "benzoic acid analogue" nomenclature used to denote the same compound in a previous publication (8).

TABLE IV

*Competitive Inhibition of Thyroxine-Like Activity of 3,5-Diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic Acid by O-Benzyl-3,5-diiodo-*DL*-tyrosine*

O-Benzyl-3,5-diiodo-DL-tyrosine	Per cent decrease in length after 72 hrs. incubation				
	3,5-Diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid per ml.				
	0 γ	2.5 γ	5.0 γ	7.5 γ	10.0 γ
γ per ml.					
0	0	29	46	50	52
10.0	0	25	42	49	52
25.0	0	21	38	42	48
50.0	0	18	34	40	44
100.0	0	13	27	33	38
200.0	0	11	17	20	29
$(I/T)_{50\%}$ weight...........		35	30	22	25
$(I/T)_{50\%}$ molar............		50	43	32	36

See the corresponding foot-notes to Table I.

No inhibition of the thyroxine-like activity of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline was observed with any of the above thyroxine antagonists even at molar ratios greater than those which reduced the activity of thyroxine to less than 50 per cent as indicated in sample data reported in Table V.

Specificity of Thyroxine Inhibition—The specificity of the inhibition of thyroxine by the type of competitive inhibitors described in previous paragraphs was tested by administering simultaneously with DL-thyroxine a number of compounds, including structurally related, goitrogenic, and polyfunctional substances. The thyroxine was used at a concentration of 0.50 γ per ml. which, when given alone, resulted in a decrease in length of 45 ± 5 per cent in 3 days incubation at 31° ± 1°. A compound was concluded to be without inhibitory effect when the thyroxine response was not altered by more than a 10 per cent decrease in length. The maximum concentration of potential inhibitor was determined by its toxicity, by its water solubility at pH 8.0 ± 0.5, and finally by a level just below activity in case the compound itself exerted thyroxine-like activity (*e.g.*, *N*-acetyl thyroxine, etc.). A high specificity for thyroxine antagonism was indicated by the fact that none of the following compounds inhibited the effect of thyroxine when tested at the maximum possible molecular ratios listed:

Table V

Absence of Effect of Thyroxine Inhibitors on Thyroxine-Like Activity of 3,5-Diiodo-4-(4'-hydroxyphenoxy)-aniline

Experiment No.	Inhibitor		3,5-Diiodo-4-(4' hydroxyphenoxy)-aniline	Molar ratio*	Per cent decrease in length†
		γ per ml.	γ per ml.		
12	*O*-Benzyl-*N*-acetyl-3,5-diiodotyrosine	0	1.0	0	55
		250	1.0	200	55
		500	1.0	400	55
	4-Benzyloxy-3,5-diiodobenzoic acid	0	1.0	0	55
		5.0	1.0	4.5	56
		7.5	1.0	6.8	54
19	*O*-Benzyl-3,5-diiodotyrosine	0	1.0	0	45
		10	1.0	8.6	42
		25	1.0	22	45
		50	1.0	43	45
		100	1.0	86	45
		200	1.0	172	45
		0	2.0	0	50
		10	2.0	4.3	45
		25	2.0	11	45
		50	2.0	22	42
		100	2.0	43	52
		200	2.0	86	45

* Molar ratio of inhibitor to thyroxine-like, active compound.

† The incubation time in these experiments was 80 ± 5 hours.

N-acetyl-DL-thyroxine, 10; 3,5-diiodo-4-(4'-hydroxyphenoxy)-benzoic acid, 15; 3,5-diiodo-4-hydroxybenzoic acid, 1000; 3,5-difluoro-4-methoxyphenol, 440; 3,5-dichloro-4-anisic acid, 350; 2-amino-3,5-diiodobenzoic acid, 100; L-tyrosine, 2000; *N*-acetyl-3,5-diiodo-L-tyrosine, 1600; 4-fluorobenzoic acid, 5000; 4-anisic acid, 500; 3-hydroxybenzoic acid, 280; salicylic acid, 1700; 4-aminobenzoic acid, 2830; 4-iodobenzoic acid, 1560; 4-anisidine, 3150; sulfanilic acid, 1350; sulfanilamide, 2250; benzoic acid, 6300; benzyl alcohol, 1800; 2-thiouracil, 2400; 2-mercaptoimidazole, 1550; ascorbic acid, 1770; sodium iodide, 5000; malonic acid, 750; acetonitrile, 350.

DISCUSSION

Table VI summarizes the experiments involving all pairs of thyroid-active and antagonistic compounds. Considerable modification of the structure of thyroxine has resulted in the production of potent competitive antagonists.

TABLE VI

Summary of Inhibition of Thyroxine-Like Active Compounds in Amphibia

Thyroxine-like compound	Inhibitor*	$(I/T)_{50\%}$†	Comment
DL-Thyroxine	A	37	Competitive inhibition, see Table II, Fig. 1
"	B	550	Competitive inhibition, see Table I
"	C	8	Competitive inhibition, see Table III
"	D	75	Competitive inhibition
"	E	150	" "
Glycine homologue of thyroxine	B	650	Probably competitive inhibition
" "	C	10	Probably competitive inhibition
Carboxylic acid analogue of thyroxine	A	36	Competitive inhibition, see Table IV
" "	B	400	Competitive inhibition, toxicity of B prevented testing at higher ratios
" "	C	5	Competitive inhibition
3,5 - Diiodo - 4 - (4′ - hydroxyphenoxy)-aniline	A		No inhibition at 172, see Table V
"	B		" " " 400 " " "
"	C		" " " 7 " " "

* Inhibitors are designated A, *O*-benzyl-3,5-diiodo-DL-tyrosine; B, *O*-benzyl-*N*-acetyl-3,5-diiodo-L-tyrosine; C, 4-benzyloxy-3,5-diiodobenzoic acid; D, 3,5-diiodo-4-(p-nitrophenylethoxy)-benzoic acid; E, 3,5-diiodo-4-anisic acid.

† The value of (I/T) at highest inhibitor concentration is used in this column, since this value is least affected by unsaturation of the system with respect to inhibitor or thyroxine.

A most interesting finding is that these antagonists appear to have the same lack of specificity with regard to the side chain that has been previously described for thyroxine-like activity (8). Modification of the other end of the molecule, however, has very pronounced effects on both thyroxine-like activity and antagonism. If it is assumed that thyroxine acts as a coenzyme or a prosthetic group in an enzyme or family of enzymes, these facts may suggest that the thyroxine molecule associates with an enzyme by means of its side chain and exerts its function by means of the ortho-diiodohydroxyphenyl ether structure at the opposite end of the molecule. Niemann *et al.* (11, 12) likewise have suggested an active locus of the molecule by the formation of a quinoid resonance form involving the phenolic hydroxyl group and the ether oxygen. The observation that several potent antagonists of thyroxine and its acid analogues have no influence on the thyroxine-like action of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline can most readily be interpreted as an indication that this thyroid-active substance combines with different groups, perhaps of opposite polarity at physiological pH values, in the postulated enzyme system than do the thyroxine-like active compounds with acidic side chains.

In earlier work (8) it was not possible to determine whether or not certain thyroid-active compounds were independently active or were converted to thyroxine. If such compounds were converted to thyroxine, molar ratios necessary to reduce the thyroxine-like effect to one-half would be lower in the case of the less active compound. Table VI indicates the similarity of such molar ratios for thyroxine as compared to the carboxylic acid analogue. No influence on the thyroxine-like activity of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline was observed for even larger molar ratios. It is, therefore, suggested that these two compounds are not converted to thyroxine to exert their action on Amphibia.

SUMMARY

Several derivatives of 3,5-diiodotyrosine and of 3,5-diiodo-4-hydroxybenzoic acid have been prepared and observed to inhibit competitively the effects of thyroxine on amphibian metamorphosis. The respective benzyl ethers of 3,5-diiodo-4-hydroxybenzoic acid and of 3,5-diiodotyrosine were found to reduce the effect of thyroxine by 50 per cent when the molar ratio of the inhibitor to thyroxine was 8 and 37 respectively. The specificity of this inhibition was demonstrated by the inability of many other substances to produce any inhibition at much higher molar ratios.

The benzyl ethers of 3,5-diiodotyrosine and of 3,5-diiodo-4-hydroxybenzoic acid also proved to be competitive inhibitors of the thyroxine-like active compound, 3,5-diiodo-4-(3′,5′-diiodo-4′-hydroxyphenoxy)-benzoic acid. This inhibition was achieved at molar ratios similar to those obtained with thyroxine. However, these inhibitors had no effect upon the thyroxine-like activity of 3,5-diiodo-4-(4′-hydroxyphenoxy)-aniline at very high molar ratios. The fact that concentrations of inhibitors which reduced the effect of thyroxine appreciably did not alter the activity of these thyroxine-like compounds indicates that they are not converted to thyroxine for activity.

Assuming that thyroxine-like active compounds act by virtue of serving as a prosthetic group or coenzyme for some enzyme system or systems, this and other recent evidence suggests that the side chain of thyroxine serves as an enzyme-associating locus, while the orthodiiodohydroxyphenyl ether group acts as an "active" or "functional" locus of the molecule.

We are greatly indebted to Joy Levin Forrester for assistance in performing the syntheses described, and to Elizabeth V. Tukich for assistance in the biological tests.

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THE CARBOHYDRATE METABOLISM OF TRYPANOSOMA HIPPICUM*

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Investigations into the nature of carbohydrate metabolism of *Trypanosoma hippicum* have been undertaken with a two-fold purpose: (1) to gather further information concerning the comparative biochemistry of trypanosomes, and (2) to ascertain whether there are detectable metabolic differences between normal and arsenic-resistant trypanosomes.

Considerable differences seem to exist among several species of trypanosomes as regards their metabolism. Glucose is mainly converted into the 3-carbon pyruvic acid by *T. equiperdum* (1, 2) and *T. evansi* (3), while *T. lewisi* (2, 4, 5) produces the 4-carbon succinic acid, the 3-carbon pyruvic and lactic acids, and several 1- and 2-carbon fragments, apparently utilizing carbon dioxide in the process. *T. rhodesiense* (6) also forms succinic acid and a host of other products, but the R.Q. (7) is much lower than that of *T. lewisi*. Unlike other species of trypanosomes, *T. cruzi* (8) is thought to be relatively independent of sulfhydryl enzymes. Phosphorylating mechanisms for the degradation of glucose have been claimed for *T. equiperdum* (9) and *T. evansi* (3). Most trypanosomes appear to be able to utilize glycerol. Arsenic-resistant trypanosomes have been reported not to differ from normal strains with respect to the balance of substrate oxidation (2, 6).

The present report shows that *T. hippicum* resembles *T. evansi* and *T. equiperdum*. Certain features of the metabolism of this hemoflagellate may be of general biochemical interest. Work with an arsenic-resistant strain of the parasite has offered no evidence for a metabolic basis of arsenic resistance.

EXPERIMENTAL

Analytical Methods

Measurements of *gas exchange* were made manometrically at 38° with a Barcroft-Warburg constant volume respirometer by the methods of Dixon (10). *Respiratory quotients* were determined by the direct method wherein

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vessels contained potassium hydroxide in the center wells for the measurement of oxygen consumption, while duplicate vessels without potassium hydroxide were simultaneously employed for the measurement of the sum of oxygen consumption and carbon dioxide production. Initial and retained carbon dioxide was determined in control and test vessels, respectively, by the addition of acid from the side arm at the appropriate times.

Acid production was measured by the displacement of carbon dioxide from bicarbonate contained in the suspension medium, with corrections for carbon dioxide retention from the method described above.

In the experiments on the balance of substrate oxidation *glucose* was determined by the method of Nelson (11). In all other experiments the Folin-Malmros method (12) was used. *Fructose* was analyzed according to Roe (13). *Pyruvate* and *total hydrazones* were measured as outlined by Friedemann and Haugen (14), while *lactic acid* was determined by the procedure of Barker and Summerson (15) modified according to Speck, Moulder, and Evans (16).

Glycerol was measured by a method developed in this laboratory after the Hovey-Hodgins qualitative test (17). To a sample of 1.8 ml. of the trypanosome suspension were added 0.2 ml. of 20 per cent copper sulfate and 0.2 gm. of calcium hydroxide. After the solution had stood at room temperature for 2 hours, the insoluble matter was removed by centrifugation. To 1.5 ml. of the clear supernatant fluid was added 1.0 ml. of 0.1 per cent 2,4-dintrophenylhydrazine in 4 N hydrochloric acid. At the end of 10 minutes the reaction mixture was vigorously extracted with five 5 ml. portions of redistilled xylene. 1 ml. of the colorless aqueous layer was removed to a colorimeter tube to which were then added 1.0 ml. of fresh 10 per cent catechol and 3.0 ml. of concentrated sulfuric acid. The tube was then placed in a sulfuric acid bath at 140–145° for 10 minutes, and the development of a pink color ensued. At the end of the incubation period the tube was cooled in ice water. Colorimeter readings (Klett-Summerson) were made by use of a green filter having a maximum absorption at a wave-length of 540 mμ. 40 to 200 γ of glycerol may be determined by this method, and the optical density is directly proportional to the concentration of glycerol in this range. Glucose, pyruvate, and lactate give a color with the reagent; glucose is removed by the lime and pyruvate by the extraction of the dinitrophenylhydrazone. Lactate was not present in the experimental samples.

The *phosphorylated intermediates* and related esters were analyzed according to Procedure B outlined by Umbreit, Burris, and Stauffer (18), with all the analytical methods recommended by them. Glycerophosphates were determined by the method of Leva and Rapoport (19) with the modification of LePage (20). Subsequent analyses of simpler mixtures, in which one or more of phosphate, adenosine triphosphate, triose phosphate, glucose-6-phosphate, glucose, and fructose were contained, were made by the same analytical methods.

Specific enzymatic analyses and other details of experimental procedure will be described later.

Propagation and Preparation of Parasites

The parent strain of *T. hippicum* used in this investigation was kindly supplied by Dr. Malcolme H. Soule of the University of Michigan. The trypanosomes were propagated by serial transfer through Sprague-Dawley albino rats approximately 250 gm. in weight. The rats were fed *ad libitum* from a diet of a half-and-half mixture of bread and ground beef lungs and of Purina dog checkers. Transfers of the infection from rat to rat were regulated in such a manner that death of the animal occurred in 2 or 3 days.

When the infection in the rat rose above 500,000 trypanosomes per c.mm. of blood, the trypanosomes were harvested by decapitating the rat and collecting the blood in 1 ml. of heparinized Krebs-Ringer-phosphate solution, pH 7.4 (21). Differential centrifugation of 8 minutes duration (1500 R.P.M., head radius 12.5 cm.) was begun immediately. For whole cell preparations the white trypanosome layer was siphoned off into 10 ml. of the chilled experimental suspension medium and recentrifuged. Further washings were made if necessary. The trypanosome layer was then siphoned into 8 volumes of the suspending medium. Suspensions prepared in this manner contained 2 to 10 × 10^5 parasites per c.mm. Lysates were prepared by washing with chilled isotonic saline and finally siphoning into 8 volumes of distilled water.

Results

General Metabolic Properties of Whole Cell Preparations

Optimal Conditions of Concentration and Population—In the following work 3 ml. of Krebs-Ringer-phosphate solution were the selected suspension medium, and the vessels were gassed with air. Center wells contained 0.2 ml. of 20 per cent potassium hydroxide. The vessels were equilibrated for 10 minutes, and all determinations were run at 38°. No respiration of the trypanosomes was found to occur in the absence of added substrates. Furthermore, only quite stable preparations could survive, even in part, an equilibration period of 5 or more minutes if the substrate were withheld during that time. Techniques in which the addition of substrates or of parasites from the side arm was employed were thereby generally precluded; hence from the outset trypanosomes and substrate were placed together in the main compartment of the respiration vessel.

The relationship of oxygen assimilation to glucose concentration is illustrated in Fig. 1. The initial and hourly rates are seen to be nearly linearly dependent upon the glucose concentration up to about 0.003 M, after which the curve assumes plateau characteristics, the plateau of which is stable beyond concentrations of 0.07 M. Substrate analyses at the end of the experimental period and correlation of the amount of oxygen used with the available glucose showed that the glucose was always in excess; so that the relationship shown is truly a dependence upon concentration.

In order to determine suitable population densities to be employed in further experimentation, the number of trypanosomes in a vessel was varied between rather wide limits. A graphical representation of an example is given in Fig. 2 wherein data for two experimental periods of different length are plotted. For short periods the oxygen uptake was directly proportional to the number of trypanosomes, but for periods longer than 20 minutes the quantity of oxygen consumed reached a maximum and then diminished with increasing numbers of trypanosomes. Since the ascending limb of the curve was linear up to about 20 × 10^7 trypanosomes for a period as long as 90 minutes, 5 to 20 × 10^7 trypanosomes in a 3.0 ml. volume seemed to be a good working range for experiments of 1 hour's duration. In these experiments the concentration of glucose was initially 0.011 M, which is more than sufficient to support the amount of oxygen uptake recorded. The characteristics of the curve

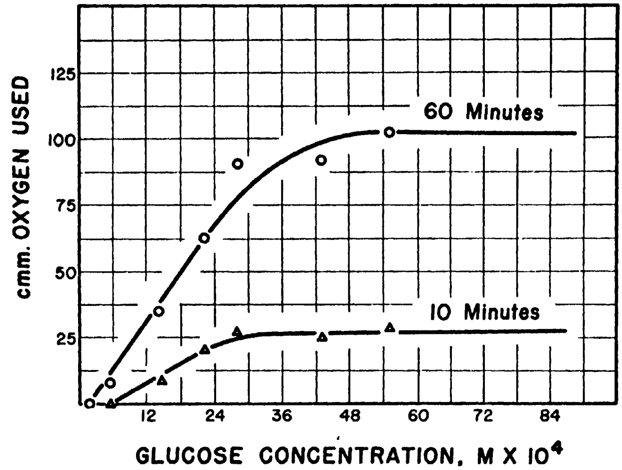

FIG. 1. The oxygen uptake of *T. hippicum* as a variable dependent upon glucose concentration. The suspension consisted of 3.0 ml. of Krebs-Ringer-phosphate solution containing 8 × 10^7 trypanosomes and glucose in the indicated concentrations. The center wells contained 0.2 ml. of 20 per cent KOH. Gas phase, air; temperature, 38°; equilibration period, 5 minutes.

could not, therefore, be attributed to limiting quantities of the substrate, nor could they be correlated with changes in pH. The rate of shaking had no effect on the shape of the curve.

Oxygen Quotients—Under the most favorable conditions of population density and of glucose concentration stable suspensions of trypanosomes were found to have an initial rate of utilization of oxygen of 15 to 25 ml. per 10^{10} trypanosomes per hour. The rates fell off rapidly from the initial value to less than 50 per cent of the initial rate in 1 hour, even in the most stable preparations. Only the initial quotients, therefore, approach the true respiratory potential of the parasites. Since the dry weight of 10^{10} trypanosomes is about 120 mg., the $Q_{O_2}^{air}$ in Krebs-Ringer-phosphate solution was about 170. The $Q_{O_2}^{air}$ (N) was about 1000. Oxygen quotients were considerably higher and steadier in mammalian plasma than in artificial media, $Q_{O_2}^{air}$ values in rat plasma reaching as high as 240.

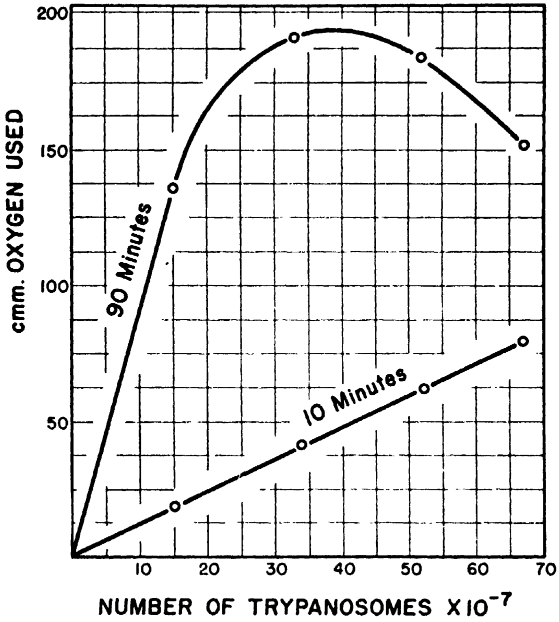

FIG. 2. The oxygen uptake of *T. hippicum* as a variable dependent upon population density. Trypanosomes in the indicated numbers were suspended in 3.0 ml. of Krebs-Ringer-phosphate containing 0.044 M glucose. The center wells contained 0.2 ml. of 20 per cent KOH. Gas phase, air; temperature, 38°; equilibration period, 10 minutes.

Respiration on Various Substrates—Nineteen compounds were tested for their ability to support the respiration of trypanosomes. Of these only glycerol seemed to be metabolized at a rate equal to or better than that of glucose. β-Glycerophosphate also gave an adequate support, being 3 times better in this respect than α-glycerophosphate. Other phosphorylated intermediates were only slightly utilized. DL-Glycerose was somewhat metabolized. Neither lactate nor pyruvate, nor any of the tested intermediates of the tricarboxylic acid cycle, was able to support the respiration of the microorganisms. None of the dicarboxylic acids accelerated the respiration of the trypanosomes when glucose was used as a substrate, nor did they enable the parasites to utilize pyruvate. Data from experiments with the various substrates are presented in Table I.

TABLE I

Oxygen Utilization of T. hippicum Maintained on Various Substrates

The complete system contained 0.127 M NaCl, 0.0051 M KCl, 0.0028 M $CaCl_2$, 0.0013 M KH_2PO_4, 0.0013 M $MgSO_4$, 0.01 M NaH_2PO_4 of pH 7.4, 0.025 M $NaHCO_3$, 5 to 20 × 10^7 whole trypanosomes, and the substrate in the indicated concentration. Total volume, 3.0 ml. The center well contained 0.2 ml. of 20 per cent KOH. Gas phase, air; temperature, 38°; experimental period, 60 minutes. A glucose control (0.04 M) was simultaneously run with each substrate for purposes of comparison.

Substrate	Concentration of substrate	Oxygen used		$\frac{Q_{O_2}\text{ substrate}}{Q_{O_2}\text{ glucose}}$
		With substrate	Glucose control	
	M	µl.	µl.	
Glycerol	0.04	363	248	1.46
α-Glycerophosphate	0.04	44	231	0.19
β-Glycerophosphate	0.04	70	108	0.65
DL-Glycerose	0.04	68	291	0.23
Glucose-6-phosphate	0.007	4	216	0.02
Hexose diphosphate	0.006	5	216	0.02
Glycogen	1 mg. per ml.	14	260	0.05

Acetate, gluconate, formate, oxalate, ethanol, malate, succinate, oxalacetate, fumarate, citrate, pyruvate, and lactate were not metabolized in concentrations of 0.02 M.

Effect of Respiratory Inhibitors—Table II shows the effect of several inhibitors on the oxygen utilization of trypanosomes suspended in Krebs-Ringer-phosphate solution with added bicarbonate when glucose was used as a substrate. The data indicate that the parasites are quite insensitive to cyanide. Even 0.1 M sodium cyanide inhibited only 87 per cent and 1.0 M inhibited 98 per cent. When glycerol was used as a substrate, very nearly the same degrees of inhibition were obtained. Precautions were taken to prevent the distillation of cyanide into the center well (18). Azide is even less effective as an inhibitor. High concentrations of fluoride were also needed to cause a severe inhibition, 0.1 M sodium fluoride being required to inhibit respiration 83 per cent. The same order of insensitivity was found with glycerol as a substrate. Trypanosomes maintained on glucose proved to be relatively sensitive to DL-glycerose, although it was found that DL-glycerose alone could be utilized as a substrate to some extent. At a concentration of 0.03 M an inhibition of 97 per cent was obtained. The sulfhydryl inhibitors iodoacetate and oxophenarsine (U. S. P.) proved to be quite potent in suppressing the oxygen utilization. Selenite and arsenate probably also exert their effect through sulfhydryl inhibition, arsenate first being reduced to arsenite. It is noteworthy that malonate exerted no effect at the concentration used.

TABLE II

Effect of Respiratory Inhibitors on Oxygen Assimilation of T. hippicum

The complete system contained 0.127 M NaCl, 0.0051 M KCl, 0.0028 M $CaCl_2$, 0.0013 M KH_2PO_4, 0.0013 M $MgSO_4$, 0.01 M Na_2HPO_4 of pH 7.4, 0.025 M $NaHCO_3$, 0.022 M glucose, 5 to 20 × 10^7 whole trypanosomes, and the inhibitor in the indicated concentration (tipped in from the side arm). Total volume, 3.0 ml. The center well contained 0.2 ml. of 20 per cent KOH, except in the case of cyanide, in which 0.1 ml. of 0.002 M KOH and 0.1 ml. of 2 M KOH were used. Gas phase, air; temperature, 38°; experimental period, 1 hour.

Inhibitor	Concentration of inhibitor	Per cent inhibition
	M	
NaCN	1×10^{-2}	36
NaN_3	1×10^{-1}	38
NaF	1×10^{-2}	15
DL-Glycerose	3×10^{-3}	48
Iodoacetate	1.6×10^{-6}	50
Oxophenarsine	4.3×10^{-6}	50
Malonate	1×10^{-2}	0
Na_2SeO_3	1×10^{-2}	38
Na_2HAsO_4	1×10^{-2}	78

Vitamins and Cofactors—None of calcium pantothenate, riboflavin, biotin, thiamine chloride, nicotinic acid, nicotinamide, sodium ascorbate, or *p*-aminobenzoic acid had any effect on the respiration of the whole cell preparations when glucose was used as a substrate, nor did adenosine triphosphate, triphosphopyridine nucleotide, diphosphopyridine nucleotide, glutathione, either singly or in combination.

Balance of Substrate Utilization

In order to determine the carbon balance of glucose and glycerol oxidation, the substrate and oxygen were initially and terminally measured, along with the simultaneous determination of several possible conversion products. Pyruvate, lactate, total hydrazones, total acid, and glycerol were analyzed. Respiratory quotients were also determined. The molar ratios between the various pairs of reagents and products of the transformation are presented in Table III together with the respiratory quotients. Lactate is also shown among the substrates, although the data indicate that it is not metabolized.

TABLE III

Balance of Substrate Utilization in T. hippicum

The complete system contained 0.127 M NaCl, 0.0051 M KCl, 0.0028 M $CaCl_2$, 0.0013 M KH_2PO_4, 0.0013 M $MgSO_4$, 0.01 M NaH_2PO_4 of pH 7.4, 0.025 M $NaHCO_3$, 5 to 20 × 10^7 whole trypanosomes, and the substrate in the indicated concentration (tipped in from the side arm). Total volume, 3.0 ml. The center well contained 0.2 ml. of 20 per cent KOH, except when CO_2 production was being measured. For aerobic trials pure oxygen was used, except when CO_2 was being measured, in which case 95 per cent O_2-5 per cent CO_2 was used. For anaerobic trials 95 per cent N_2-5 per cent CO_2 was used. Temperature, 38°; experimental period, 60 minutes.

Substrate	Concentration	Molar ratio								R.Q.
		Oxygen / Substrate	Total acid / Oxygen	Total acid / Substrate	Total hydrazones* / Substrate	Pyruvate / Oxygen	Pyruvate / Substrate	Glycerol / Substrate	Lactate† / Substrate	
Aerobic										
	M									
Glucose....	0.01–0.04	0.89	2.17	1.76	1.81	2.15	1.83	0.07	0.19	0.0
"	0.0011			1.97	2.09		2.07	0.0	0.05	
Glycerol....	0.02	0.95	1.10	0.99		1.03	0.93		0.0	0.0
Lactate.....	0.01	0.0					0.005			
Anaerobic										
Glucose....	0.01–0.04			0.95			0.98	0.91	0.07	
"	0.0011						1.05	0.88		

* Expressed in terms of pyruvate equivalent.

† This is probably pyruvate interference.

It is noteworthy that an R.Q. of 0 was obtained with either glucose or glycerol as a substrate. From this and from the molar ratios shown, it appears that aerobically the transformations did not proceed beyond pyruvate. No hydrazone-forming keto acids other than pyruvic acid were found.

At high concentrations of glucose the aerobic transformation of glucose to pyruvate was not quite quantitative, the oxygen used and the pyruvate produced being roughly 10 per cent less than the theoretical values based upon the amount of glucose used. A trace of glycerol was also detected. It is felt that the lactate shown among the products in Table III was actually pyruvate, which interfered in the lactate analysis.

It was thought that the deviations from theoretical values might be the result of a more rapid anaerobic phase than the aerobic phase could sustain at concentrations above 0.01 M. In order to test this possibility the carbon balance was investigated at lower concentrations, in which case glucose became the limiting factor. Under these conditions the gas exchange was difficult to balance, but the data indicate that glucose was quantitatively oxidized to pyruvate.

Aerobically glycerol was also shown to be quantitatively converted to pyruvate. The trace of pyruvate resulting from lactate is not significant.

In the absence of oxygen glucose is nearly quantitatively degraded to pyruvate and glycerol.

The effect of oxophenarsine upon the carbon balance was investigated, but no significant alteration in the pattern of molar ratios was noted, even under conditions of rather severe inhibition.

Glycolysis in T. hippicum

Fractionation of Phosphorylated Intermediates—Trypanosomes were thrice washed in cold isotonic saline solution and frozen for subsequent analysis. In Table IV the data are presented as micromoles of the stated compound per gm. of the frozen packed trypanosomes. 1 gm. of the trypanosomes may be considered to be roughly equivalent to 10^{10} microorganisms. The total acid-soluble phosphorus found in the parasites amounted to 20.4 μM per gm. Adenine derivatives and other labile compounds were present in only small amounts, while phosphoglyceric acids and glucose-1-phosphate were absent. The predominant organic phosphorus compounds were triose phosphates, glycerophosphates, and glucose-6-phosphate.

Hexokinase in T. hippicum—The presence of hexokinase was tested by oxygen uptake. The method is an adaptation of a principle employed by Slein[1] who measured hexokinase activity spectrophotometrically by following the reduction of triphosphopyridine nucleotide (TPN) by Robison ester dehydrogenase (*Zwischenferment*). Since this requires a considerable amount of TPN, it was deemed practical to measure oxygen uptake, instead, by including yellow enzyme in the system. Only catalytic amounts of TPN were thereby required. The results of tests carried out on homogenates of the parasites are shown in Table V. Hexokinase activity is shown by an appreciable oxygen uptake which is dependent upon glucose, adenosine triphosphate (ATP), TPN, and *Zwischenferment*. In confirmation of this, chemical analyses show that hexose diphosphate was formed and that considerable amounts of ATP were destroyed. The trypanocide, oxophenarsine, was included to show the sensitivity of the enzyme to sulfhydryl inhibitors.

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[1] Slein, M. J., personal communication.

Trypanosomes themselves were not found to contain *Zwischenferment* when assayed by the method of Negelein and Gerischer (22), although the data of Table V suggested such a possibility.

TABLE IV

Phosphorylated Esters and Related Compounds in T. hippicum

Fraction	Compound*		Per cent phosphorus of fraction
		µM per gm.	
Barium-insoluble	Inorganic	3.9	50
	Hexose diphosphate	0.10	2.7
	Phosphoglyceric acid	0.0	0.0
	ATP + ADP	0.03	1.2
Total			54
Barium-soluble-alcohol-insoluble	Phosphocreatine	0.6	8
	Phosphopyruvate	0.6	8
	Glucose-1-phosphate	0.0	0.0
	Glucose-6-phosphate	1.2	16.0
	Fructose-6-phosphate	0.07	0.9
	Triose phosphate	2.4	30
	Pentose "	0.07	0.9
	Adenylic acid	0.18	2.5
	DPN + TPN	0.05	2
	Glycerophosphates	2.16	29.5
Total			98

* ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

Adenosinetriphosphatase—Table V shows that a considerable amount of ATP was split in the absence of glucose. That this may be attributed to specific adenosinetriphosphatase determined by assay according to DuBois and Potter (23), the results of which are given in Table VI. Calcium activated the enzyme, as was expected, but magnesium activated the enzyme to even a greater extent at the usually inhibiting concentration of 0.009 M. Fluoride had no effect in the absence of activating ions. In order to test the specificity of the enzyme, α- and β-glycerophosphates were included as controls; no phosphate was liberated therefrom.

Aldolase—Lysates of the microorganisms were able to convert hexose diphosphate to triose phosphates. The system of Herbert *et al.* (24) was not satisfactory in this respect, and the pH of the medium, consequently,

TABLE V

Hexokinase Activity As Measured by Oxygen Uptake in Homogenates of T. hippicum

The complete system contained 0.017 M $NaHCO_3$, 0.5 mg. of KCN, 80 mg. of old yellow enzyme, 7.5 of *Zwischenferment*, 0.012 M glucose, 0.005 M ATP, 12 γ of TPN, 0.01 M $MgCl_2$, and 0.025 M NaF. One tube contained 6.0×10^{-4} M of oxophenarsine. Total volume, 4.0 ml. A homogenate corresponding to 1×10^8 trypanosomes was added from the side arm. Gas phase, 100 per cent O_2. The center well contained 0.2 ml. of 20 per cent KOH. Temperature, 38°. The experimental period was 60 minutes. The reaction was terminated by the addition of 0.1 ml. of 100 per cent trichloroacetic acid.

System	Glucose used	Oxygen used	ATP* split	Hexose diphosphate formed
	μM	μM	μM	μM
Complete system	14.4	5.4	9.9	5.4
Without ATP	5.8	1.1		0.0
" glucose		0.4	5.8	
" TPN	14.2	1.1	9.8	7.2
" magnesium	14.9	6.1	9.2	5.1
" *Zwischenferment*	14.3	1.8	9.9	8.4
Oxophenarsine added	7.2	3.3	8.8	2.4

* Calculated on the basis that 1 mole of ATP will donate 2 atoms of phosphorus.

TABLE VI

Adenosinetriphosphatase in T. hippicum

The experimental system consisted of 0.75 ml. containing in final concentration 0.0077 M barbital buffer of pH 7.4, 0.0019 M ATP (or 0.0032 M α- and β-glycerophosphate), approximately 5×10^7 thrice washed homogenized trypanosomes, and either 0.0061 M $CaCl_2$ or 0.009 M $MgCl_2$ or 0.015 M NaF as indicated. Temperature, 37°. The reaction was stopped at the end of 15 minutes by the addition of 0.1 ml. of 100 per cent trichloroacetic acid.

System	Phosphorus	ATP split	ATPase (10^7 trypanosomes = 1 mg.)
	γ	*per cent*	*units per mg.*
Control	30	17	6.0
With $CaCl_2$	78	43	15.6
" $MgCl_2$	130	72	26.0
" NaF	30	17	6.0
α-, β-glycerophosphate control	0.8		

was raised to 8.0. The system contained 0.01 M borate buffer of pH 8.0, 0.01 M hexose diphosphate (added from the side arm), 0.033 M potassium fluoride, 0.06 M potassium cyanide, and 0.5 ml. of a lysate of thrice washed trypanosomes (about 6.4 × 10^8 trypanosomes). The total volume was 3.0 ml. Nitrogen was used for gassing the vessels. An experimental period of 60 minutes at a temperature of 38° was concluded by the addition of 0.2 ml. of 100 per cent trichloroacetic acid to each vessel. In this system 5.6 μM of hexose diphosphate were used and 8.2 μM of triose phosphate were formed.

Triose Phosphate Dehydrogenase and Coupled Oxidation-Reduction—The system used in this analysis was adapted from one employed by Speck and Evans on extracts of malaria parasites (25). The presence of aldolase indicated that hexose diphosphate could be used for the substrate, provided that aldolase is not the rate-determining factor in the sequence. Triose phosphate dehydrogenase activity is shown by the data in Table VII, with an appreciable evolution of carbon dioxide which is dependent upon the presence of hexose diphosphate and diphosphopyridine nucleotide and which shows some activation by magnesium ions. The presence of pyruvate, however, did not seem to be necessary for the optimal functioning of the system; evidently, dihydroxyacetone phosphate can oxidize DPNH rapidly enough to keep pace with the dehydrogenase.

TABLE VII

Triose Phosphate Dehydrogenase in T. hippicum

The complete system of 3.0 ml. contained in final concentration 0.025 M $NaHCO_3$, 0.01 M hexose diphosphate (tipped from the side arm), 0.0001 M diphosphopyridine nucleotide, 0.005 M $MgCl_2$, 0.033 M KF, 0.012 M glucose, 0.00066 M ATP (tipped in from the side arm), 0.01 M phosphate (pH 7.4), 0.012 M lithium pyruvate, and 0.5 ml. of a homogenate of thrice washed trypanosomes (about 2.5 × 10^8 trypanosomes). Gas phase, 95 per cent N_2-5 per cent CO_2. Temperature, 38°; experimental period, 60 minutes.

System	CO_2
	μM
Complete system	7.3
Without hexose diphosphate	0.3
" DPN	1.1
" $MgCl_2$	5.0
" pyruvate	7.6

The coupling of oxidation-reduction with phosphorylation is illustrated in Table VIII. In the absence of phosphate there was a marked diminution in the production of carbon dioxide. The activity of the system was also diminished by withholding either ATP or glucose. In the presence of arsenate, however, neither phosphate, glucose, nor ATP was required.

Triose phosphate dehydrogenase is sensitive to sulfhydryl inhibitors. Since oxophenarsine is chemotherapeutically of more interest than iodoacetate, the former was used to test the sensitivity of the system to sulfhydryl inhibitors. In order to avoid equivocalities, the arsenate system was chosen in preference to the more complicated coupled system of Table VII. The data show a 53 per cent inhibition of triose phosphate dehydrogenase activity by oxophenarsine at the low concentration of 1.2×10^{-5} M.

Glycerol and Glycerophosphate Dehydrogenases—Since trypanosomes utilize and produce glycerol with facility, it seemed only likely that either or both glycerol or glycerophosphate dehydrogenases were operative. Glycerol, α-glycerophosphate, and β-glycerophosphate were tested for their ability to reduce 2,6-dichlorophenol indophenol in the presence of trypanosome dialysates. After lysis trypanosomes were dialyzed against isotonic sodium chloride for 6 hours at 0°. The components of the Thunberg system are given in Table IX. The reduction of the dye was followed colorimetrically in the Klett-Summerson instrument fitted with a filter of maximum absorption at 660 mμ. The data show that all three substrates were capable of promoting the reduction of the dye at approximately the same rapid rate. Surprisingly, the reduction proved to be independent of coenzyme I.

TABLE VIII

Coupled Oxidation-Reduction in T. hippicum

The complete system is identical with the above system described for triose phosphate dehydrogenase (Table VII). In some vessels 0.001 M Na_2HAsO_4 replaced glucose, ATP, and phosphate. In one vessel containing arsenate 1.2×10^{-5} M oxophenarsine was added (tipped in from the side arm).

System	CO_2
	μM
Complete system	7.3
Without phosphate	1.1
" ATP	4.8
" glucose	4.0
Arsenate system	5.1
" " + oxophenarsine	2.4

Phosphorylation of Glycerol—Although it was shown that non-phosphorylated glycerol could be dehydrogenated, it seemed plausible that glycerol could enter the metabolic sequence of events through a phosphorylative mechanism similar to the mode of entry of glucose into glycolysis. In order to test this possibility the system of Colowick and Kalckar (26) was used, except that glycerol was used in place of glucose. The results are expressed in Table X. Carbon dioxide evolution was clearly dependent upon both glycerol and ATP and may be taken as evidence of the phosphorylation of glycerol.

Alkaline Phosphatase—At pH 7.3 α- and β-glycerophosphates were not hydrolyzed (Table VI). Alkaline phosphatase, however, could be demonstrated at pH 9.8 by employing the same substrates. The results are presented in Table XI. While activity was quite definite, it was not marked. Magnesium ions appeared to activate the enzyme only about 22 per cent at the concentration used.

Table IX

Glycerol and Glycerophosphate Dehydrogenases in T. hippicum

The complete system contained 0.01 M barbital buffer of pH 7.4, 0.02 M NaF, 100 γ per ml. of sodium dichlorophenol indophenol, a dialysate of approximately 2 × 10^8 trypanosomes, and either 0.04 M α-glycerophosphate, 0.04 M β-glycerophosphate, or 0.04 M glycerol. Total volume, 5.0 ml.; temperature, 23°. The *rate* is the 5 minute change in scale reading of the Klett-Summerson colorimeter fitted with a 660 mμ filter.

Substrate	Rate
α-Glycerophosphate	90
β-Glycerophosphate	95
Glycerol	80
No substrate	10

Table X

Phosphorylation of Glycerol by T. hippicum

The complete system contained 0.03 M $NaHCO_3$, 0.033 M NaF, 0.01 M $MgCl_2$, 0.049 M ATP, 0.04 M glycerol, and a lysate of 2 × 10^8 thrice washed trypanosomes. Total volume, 3.0 ml.; gas phase, 95 per cent N_2-5 per cent CO_2. Measurements were made over a 30 minute period at a temperature of 38°.

System	CO_2
	μM
Complete system	5.0
Without glycerol	1.5
" ATP	0.0
" $MgCl_2$	5.8

Other Enzymatic Assays

It has been shown above that lactate was not assimilated by trypanosomes, nor could the parasites produce lactate from pyruvate. Furthermore, pyruvate appeared to be completely dispensable in the triose phosphate dehydrogenase system. Considerable doubt, therefore, existed as to the presence of lactic dehydrogenase in *T. hippicum*. In this respect the Thunberg system used by Speck and Evans (25) for the assay of the enzyme was employed, and the enzyme was found lacking in homogenates of the parasites.

Homogenates of the microorganisms were tested also for cytochrome oxidase and succinic dehydrogenase by the method of Schneider and Potter (27). These two enzymes were not demonstrable by this method. Malic dehydrogenase could not be demonstrated by the method of Potter (28). Nor could these latter two dehydrogenases be shown by Thunberg techniques according to the methods of Gale and Stephenson (29) and Tam and Wilson (30).

Catalase was assayed in order to determine whether trypanosomes are deficient in iron-containing enzymes other than the cytochromes. By the method of von Euler and Josephson (31) the catalase activity was found to be no greater than the low value of 0.10.

TABLE XI

Alkaline Phosphatase in T. hippicum

The complete system contained 0.027 M borate buffer of pH 9.8, 0.005 M $MgCl_2$, 0.008 M α- and β-glycerophosphate (52 per cent α-), and a lysate of 1.4 × 10^8 thrice washed trypanosomes. Total volume, 5.8 ml.; temperature 37°; time, 1 hour.

System	Phosphorus liberated
	microatoms
Complete system	2.8
Without substrate	0.0
" $MgCl_2$	2.3

Arsenic-Resistant Trypanosomes

An oxophenarsine-resistant strain of *T. hippicum* was compared to the parent normal strain. In no respect thus far studied were any significant differences noted between normal and resistant trypanosomes, except that among the respiratory inhibitors oxophenarsine was required by whole resistant trypanosomes in a higher (10-fold) concentration to bring about 50 per cent inhibition of oxygen uptake. Respiratory inhibition, substrate utilization, and carbon balance were otherwise alike in the two strains. Likewise, the resistant strain lacked cytochrome oxidase, succinic, malic, and lactic dehydrogenases. The enzymes of glycolysis have not yet been studied in resistant trypanosomes.

DISCUSSION

From the evidence presented in Table III it appears that both glucose and glycerol are quantitatively oxidized to pyruvic acid as in the following equations.

$$C_6H_{12}O_6 + O_2 \rightarrow 2CH_3COCO_2H + 2H_2O$$
$$C_3H_5(OH)_3 + O_2 \rightarrow CH_3COCO_2H + 2H_2O$$

Anaerobically the transformation is

$$C_6H_{12}O_6 \rightarrow C_3H_5(OH)_3 + CH_3COCO_2H$$

In this respect *T. hippicum*, *T. equiperdum*, and *T. evansi* are similar and may be distinguished as a group from species with more complicated metabolic schemes. It cannot be assumed, however, that these three species are identical with respect to their carbohydrate metabolism. While it is generally stated that *T. equiperdum* quantitatively oxidizes glucose to pyruvate, the data of Reiner, Smythe, and Pedlow show that the quantity of total acids produced falls short of the theoretical by some 10 or 12 per cent. Only 50 to 75 per cent of the titratable acid was demonstrable as pyruvic acid. In mouse blood *T. evansi* does not quantitatively produce pyruvic acid from glucose and it is suggested that some pyruvate is decarboxylated (3). Certain other differences apparently exist among the three species. *T. hippicum* does not contain as much acid-soluble phosphorus as does *T. equiperdum* (32). *T. evansi* is quite sensitive to fluoride, while *T. equiperdum* (33) and *T. hippicum* are not. Fluoride also has dissimilar effects upon the adenosine triphosphatase of *T. hippicum* and *T. equiperdum*, inhibiting only that of the latter (9).

Phosphorylative glycolysis is frequently taken for granted in animal organisms. The uncertain position of Reiner, Smythe, and Pedlow (2) gave rise to the general belief that phosphorylation does not occur in trypanosomes. Chen and Geiling, however, presented some evidence of phosphorylation in *T. equiperdum* (9), and Marshall demonstrated phosphorylated intermediates in the blood of mice infected with *T. evansi* (3). Although the present report shows that phosphorylative mechanisms for the degradion of glucose and glycerol exist in *T. hippicum*, an explanation of the relative insensitivity of the living parasite to fluoride remains obscure, as does an explanation of the paradoxical situation wherein DL-glycerose may be utilized as a substrate and yet effectively inhibit the utilization of glucose.

With respect to the relative distribution of the phosphorylated intermediates *T. hippicum* and *T. evansi* are similar, except that the latter is reported to produce considerably more ATP. It is uncertain what criteria were employed by Marshall as a measure of ATP. We have found the 7 minute phosphorus content of *T. hippicum* to be much greater than can be accounted for by adenosine phosphates and other known acid-labile phosphates, and the figure given in Table IV is calculated from the limiting quantity of nitrogen found in the barium-insoluble fraction. It is to be expected from the method of preparation of the parasites for fractionation that the adenosine triphosphate and adenosine diphosphate content should be low, but it would also be expected that the adenylic acid or adenine content would be proportionately higher. Since this was not the case, it may be suggested that the living trypanosome contains ATP in small quantities, but this of course does not imply a functional deficiency in the compound. A similar situation occurred with the pyridine nucleotides, wherein no correlation could be found between the amount of adenine nitrogen, pentose, and nicotinamide. The values reported are based upon nicotinamide, which was found in limiting amounts.

The demonstration of a phosphorylative mechanism for the metabolic initiation of glycerol is significant but not unexpected. Gunsalus and Umbreit (34) have shown that *Streptococcus faecalis* phosphorylates glycerol. It is interesting that the two glycerophosphates and glycerol itself can be dehydrogenated with nearly equal facility. It is important to note that the glycerophosphate dehydrogenase found was neither DPN-linked nor cytochrome-linked, which two facts distinguish this enzyme from glycerophosphate dehydrogenases I and II, respectively. The same anomaly has been reported for *S. faecalis* (34).

The manner in which electron transfer is accomplished in the absence of cytochrome oxidase requires elucidation. Cytochrome by-passes are not unknown, but they are generally too slow to account for the extremely rapid turnover found in trypanosomes. The cytochrome-deficient *S. faecalis* also has a high Q_{O_2}, but it differs from trypanosomes in that hydrogen peroxide is an end-product (34). In view of the extreme motility of trypanosomes it would appear that the electron transfer system is fairly efficient from the standpoint of making energy available for useful work.

The over-all metabolic scheme, however, is inefficient, since it utilizes less than 15 per cent of the available free energy of glucose. Since no mechanism exists for the dissimilation of pyruvate, the parasite becomes dependent upon the host to remove this waste and prevent autointoxication. The host is thus able to salvage a large proportion of the energy originally stored in glucose. This may partially explain why a rat, for instance, will not show signs of infection until about 10 per cent of the blood volume is occupied by the parasites.

Marshall has concluded that arsenicals act on trypanosomes by inhibiting hexokinase (3). In this investigation both hexokinase and triose phosphate dehydrogenase were found to be sensitive to oxophenarsine, but the latter enzyme was considerably the more sensitive. On the other hand, if the latter enzyme were primarily affected in the whole trypanosome, disturbances in the balance of substrate oxidation might be expected. This, however, was not the case.

Although no differences were found between normal and arsenic-resistant trypanosomes in properties in which they were compared, this approach to the solution of the problem is by no means closed. The critical experiment of comparing the arsenic sensitivity of the sulfhydryl enzymes of the two strains should contribute to a clarification of the problem.

SUMMARY

1. *Trypanosoma hippicum* was shown to oxidize glucose and glycerol quantitatively to pyruvic acid. Anaerobically glucose was found to be converted into glycerol and pyruvic acid.

2. Glycolysis was found to conform to the Meyerhof-Cori-Embden scheme, except that lactate cannot be formed. Specific enzymatic analysis confirmed the presence of hexokinase, adenosinetriphosphatase, aldolase, triose phosphate dehydrogenase with coupled oxidation-reduction, glycerol and α- and β-glycerophosphate dehydrogenases, and an alkaline phosphatase.

3. Fractionations of the acid-soluble phosphorus were made. The content of phosphorylated intermediates was found to be low, residing principally in glucose-6-phosphate, triose phosphate, and glycerophosphates.

4. Specific enzymatic analyses were unable to reveal the presence of cytochrome oxidase, succinic dehydrogenase, malic dehydrogenase, and lactic dehydrogenase. The catalase content was found to be quite low. No evidence of a tricarboxylic acid cycle was found.

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DESTRUCTION OF 3-β-HYDROXY 17-KETOSTEROIDS DURING HYDROLYSIS WITH HYDROCHLORIC ACID*

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Acid hydrolysis has long been used to free the urinary 17-ketosteroids from the water-soluble combinations in which they are excreted. Both hydrochloric acid (13, 5, 11) and sulfuric acid (15)[1] have been employed for this purpose. The acid treatment, while effecting relatively complete hydrolysis, may also result in destruction (13, 5, 6, 3, 16), chlorine substitution (2), dehydration (20, 4, 12), and in stereoisomeric transformations (6, 16).

Attempts to obviate the destructive action of acid hydrolysis have been made by Talbot and his associates. They described an enzymatic hydrolytic procedure (18) and also a method of hydrolysis utilizing barium chloride at pH 5.8 (19). Their studies were conducted on urine from patients with adrenal cortical carcinoma and adrenal cortical hyperplasia (18). We have investigated the $BaCl_2$ hydrolysis of the conjugated 17-ketosteroids in specimens of normal male urine and have obtained by this method a much higher β (C_3 hydroxyl cis to the C_{10} methyl) fraction than is usually reported for normal urine. The experiments reported below further indicate that a large proportion of the β-17-ketosteroids is changed to an "α" fraction (no cis C_3 hydroxyl group) by the usual HCl hydrolytic technique.

Methods

Colorimetric Determination of 17-Ketosteroids—The 17-ketosteroid content of the urine extracts was measured by the Zimmermann reaction essentially as modified by Holtorff and Koch (9).

Preparation of Urine Extracts for Color Assay—After hydrolysis, the free 17-ketosteroids were extracted from the urine by the procedure of Talbot and Eitingon (18), CCl_4 being used as the extracting agent instead of ethyl

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[1] Samuels, L. T., personal communication (1947).

acetate. The ketonic fraction was removed by the Pincus and Pearlman modification (14) of the Girard method and then separated into "α" and β fractions by the digitonin fractionation method of Frame (7).

Hydrolysis of Conjugated 17-Ketosteroids. (*a*) *With Hydrochloric Acid*—The procedure of Talbot, Butler, MacLachlan, and Jones (16) was followed in which concentrated HCl is added to the urine in the proportion of 15 per cent by volume, and the mixture boiled under a reflux for 10 minutes.

(*b*) *With Barium Chloride*—The barium chloride technique described by Talbot and his coworkers (18, 19) was used as such or slightly modified. Their procedure requires the extraction of the urine at pH 7.0 with normal butyl alcohol, and washing of the butanol extracts with N NaOH and then with N NaAc buffer (pH 5.8). The residue obtained by removal of the butyl alcohol *in vacuo* is then dissolved in 0.1 N NaAc buffer (pH 5.8) and heated with $BaCl_2$ in a boiling water bath for 4 hours. After cooling, it is extracted with CCl_4 and fractionated for assay of the 17-ketosteroid fractions in the usual manner.

EXPERIMENTAL

Total Amount of Free 17-Ketosteroids Obtained Following $BaCl_2$ Treatment of Butanol Extracts of Norman Male Urine As Compared with Amount Obtained Following HCl Treatment—Butanol extracts of a number of normal male urine batches were subjected to the HCl and $BaCl_2$ hydrolytic techniques and the amounts of free 17-ketosteroids released by these methods were determined. It was found that an average of 40 per cent of the 17-ketosteroids which were in the butanol extracts appeared in the CCl_4 extract after treatment by the $BaCl_2$ procedure, as compared with the amount obtained after treatment with HCl.

The lower 17-ketosteroid values obtained by the $BaCl_2$ hydrolytic treatment is not due to a destruction of these compounds, but is due rather to a failure of liberation of them from their conjugates. This is shown by the recovery of the remainder of the 17-ketosteroids after HCl hydrolysis of the aqueous solutions remaining following the $BaCl_2$ treatment (non-$BaCl_2$-hydrolyzable 17-ketosteroids). Typical results are shown in Table I.

Distribution of 17-Ketosteroids Obtained Following $BaCl_2$ Treatment into "α" and β Fractions As Compared with That Obtained Following HCl Treatment—Butanol extracts of normal male urine were prepared and hydrolyzed with $BaCl_2$ and HCl as indicated above. Control urine specimens were also hydrolyzed directly with HCl, and the concentration of "α"- and β-17-ketosteroids determined in each case. The average results of a number of experiments are graphically represented in Fig. 1. The 3-β-hydroxy 17-ketosteroids in the $BaCl_2$-hydrolyzable fraction represented approximately 25 per cent of the 17-ketosteroids in the butanol extracts prepared in this manner. Since only 43 to 68 per cent (average 58 per cent) of the total 17-ketosteroids present in the urine are removed in butanol extracts

TABLE I

17-Ketosteroids Released from Their Conjugates in Butanol Extracts by $BaCl_2$ and HCl Hydrolysis

Urine batch	$BaCl_2$-hydrolyzable 17-ketosteroids (*A*)	Non-$BaCl_2$-hydrolyzable 17-ketosteroids (*B*)	(*A*) + (*B*)	HCl treatment of BuOH extract
	mg.	*mg.*	*mg.*	*mg.*
C	1.7	2.6	4.3	4.0
F	3.8	4.3	8.1	7.9

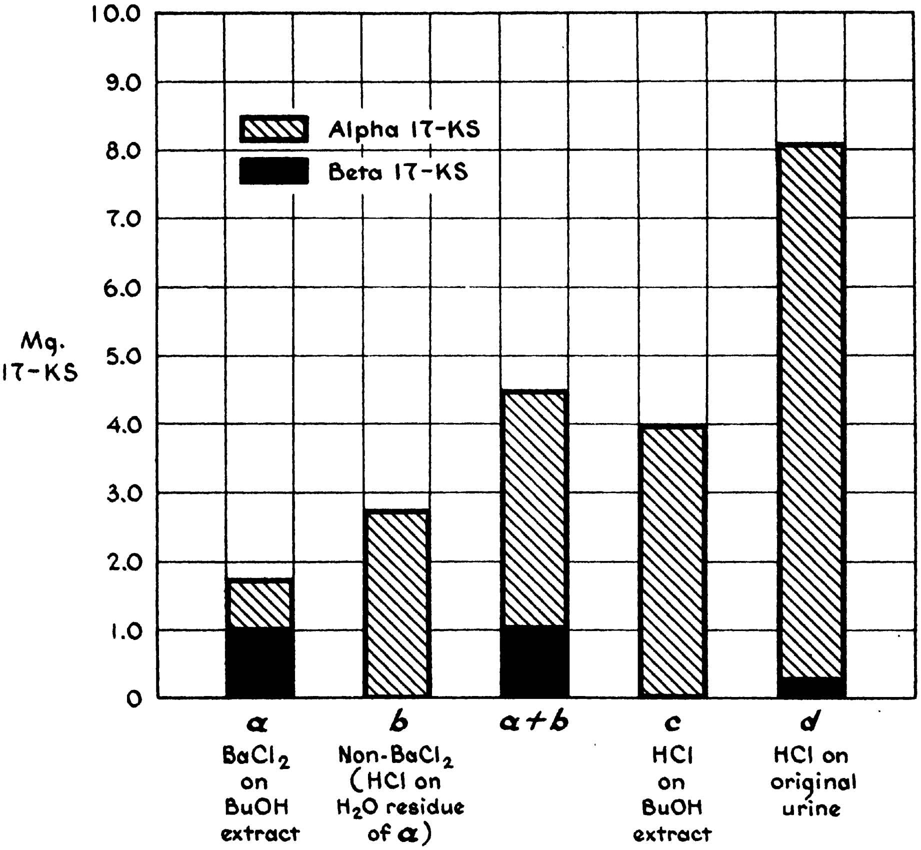

FIG. 1. Comparison of "α-" and β-17-ketosteroids obtained by the $BaCl_2$ and HCl hydrolytic techniques.

prepared at pH 7.0, this β fraction represents 11 to 16 per cent (average 13.38 per cent) of the total 17-ketosteroid content of the urine. While extraction of the urine at pH values less than 3.0 with butyl alcohol results in a quantitative transfer of the conjugated 17-ketosteroids to the butanol, the subsequent amounts of "α"- and β-17-ketosteroids released by $BaCl_2$ remain unchanged. Acid hydrolysis applied directly to the urine yielded a 3-β fraction of about 4.5 per cent of the total 17-ketosteroid content.

Conversion of β-17-Ketosteroids to "α"-17-Ketosteroids by HCl—The greater yield of 3-β-hydroxy 17-ketosteroids liberated in the $BaCl_2$ hydrolysis experiment than in that involving HCl treatment of the urine could be explained as due to (*a*) a conversion of 3-"α"-hydroxy compounds into 3-β compounds by the $BaCl_2$ or (*b*) a destruction of 3-β-hydroxy compounds by the acid. The correctness of the latter alternative was shown by the following experiments: (1) A 17-ketosteroid fraction obtained after HCl hydrolysis was subsequently subjected to the $BaCl_2$ treatment. (2) A 17-ketosteroid fraction obtained after $BaCl_2$ hydrolysis was subsequently subjected to the HCl treatment. The effects of these second hydrolyses on the distribution of the "α"- and β-17-ketosteroids were determined in both experiments and are shown in Table II, Experiments 1 and 2 respectively. It is seen that the β fraction of the $BaCl_2$ hydrolysate is considerably reduced by subsequent HCl treatment, while subsequent $BaCl_2$ treatment has no effect on the amount of β-ketosteroids in a HCl hydrolysate. These experiments thus indicated that the differences in the amounts of "α"- and β-17-ketosteroids of normal male urine obtained following $BaCl_2$ and HCl

TABLE II

Effects of HCl and $BaCl_2$ Procedures on Stereoisomerism of 17-Ketosteroids

Experiment No.	Urine batch	Hydrolytic treatment	DHA added	"α" fraction obtained	"β" fraction obtained	Change in "α" fraction over control*	Change in "β" fraction over control*
			mg.	*mg.*	*mg.*	*mg.*	*mg.*
1A	C	HCl	0.0	8.8	0.1		
1B		" followed by $BaCl_2$	0.0	8.2	0.1	−0.6	0
2A	C	$BaCl_2$	0.0	1.1	0.5		
2B		" followed by HCl	0.0	1.2	0.0	+0.1	−0.5
3A	E	HCl on urine	0.0	9.0	0.7		
3B			3.2	10.3	1.9	+1.3	+1.2
4A	E	HCl on BuOH extract	0.0	4.2	0.2		
4B			3.2	4.6	1.0	+0.4	+0.8
5A	E	$BaCl_2$ on BuOH extract	0.0	1.4	0.6		
5B			3.2	1.2	3.7	−0.2	+3.1

* Changes of 0.10 mg. or less are not considered significant in these procedures.

treatment respectively is due to the damaging effects of HCl on the β-17-ketosteroid fraction.

This difference in the effect of the $BaCl_2$ and HCl treatments on the C_3 isomerism was also confirmed with a pure 3-β-hydroxy 17-ketosteroid (dehydroisoandrosterone or DHA). 3.2 mg. of DHA were added to three portions of pooled male urine and subjected to the hydrolytic treatments as indicated in Table II, Experiments 3, 4, and 5. Control assays on urine samples to which no DHA had been added were carried out in each case, and the concentration of "α"- and β-17-ketosteroids in the hydrolysates determined in each case. It was found that where the acid treatment was effected directly on the urine, approximately 41 per cent of the added DHA appeared as the "α" fraction, while 38 per cent was recoverable in the 3-β fraction. When the acid treatment was effected on the butyl alcohol extract, only 38 per cent could be accounted for between the "α" and β fraction two-thirds of which appeared in the β fraction. The greater loss of DHA observed on acid treatment of the butanol extract may be due to a relatively greater acidity in the aqueous hydrolytic solution as compared to the urine because of loss of urinary buffers and changes in the concentration of protective agents effected during the preparation of the butyl alcohol extract. $BaCl_2$ treatment of the butyl alcohol extract resulted in no significant loss of the DHA from the β fraction (Table II, Experiments 5A and 5B).

DISCUSSION

The values herein reported for 3-β-hydroxy 17-ketosteroids following acid hydrolysis (average 4.5 per cent) are in general agreement with the findings of most workers. Baumann and Metzger (1) report values for β-17-ketosteroids of 5 per cent or less; Rhoads, Dobriner, *et al.* (15) also report values in this range (0.6 to 2.2 per cent), while Frame (7) gives an average of 2.1 per cent. Talbot and his coworkers (17) report somewhat higher values, but their determination of the β fraction was made by an indirect method (subtraction of the determined "α" from the determined total) and, as has been pointed out by Frame (7), is subject to an error of overestimation.

Utilizing the $BaCl_2$ hydrolytic technique, the 3-β-hydroxy fraction was found to constitute 11 to 16 per cent (average 13.38 per cent) of the total 17-ketosteroid content of normal male urines studied. Approximately 40 to 60 per cent of the 17-ketosteroids which are hydrolyzed by $BaCl_2$ are 3-β compounds. The $BaCl_2$ hydrolytic method results in the liberation of only 20 to 35 per cent of the 17-ketosteroids present in the urine samples, and thus almost 75 per cent of the conjugated 17-ketosteroids in normal male urine are not hydrolyzed by the $BaCl_2$ technique. The total percentage of β-17-ketosteroids may prove to be even higher when some method of hydrolysis other than the destructive HCl method is available for the non-$BaCl_2$-hydrolyzable fraction of the urinary 17-ketosteroids.

The determination of the 3-β-hydroxy 17-ketosteroids has been found to be of some differential diagnostic value in distinguishing cases of adrenal cortical hyperplasia from cases of adrenal cortical carcinoma. Talbot and his coworkers (17) and Friedgood (8) have correlated various levels of 3-β-ketosteroids with these two syndromes. The latter investigator reports β-steroids of patients with adrenal cortical hyperplasia of less than 25 per cent of the total excretion and excretions of 30 to 70 per cent of the total as β for patients with adrenal cortical carcinoma. Kepler and Mason (10), however, have found that the β fraction of patients with adrenal cortical carcinoma is not always elevated. Two cases out of six reported could not be distinguished from hyperplasia by this criterion. The destructive action of HCl reported here must be considered in this diagnostic relation, since low β values may be obtained with this hydrolysis. It seems probable, therefore, that there is even a greater excretion of β compounds in adrenal cortical hyperplasia and carcinoma than has been heretofore reported. A possible explanation of the lack of uniformity with regard to the β fraction may lie in varying degrees of destruction in individual urine specimens.

SUMMARY

1. A comparison of the effectiveness of a $BaCl_2$ hydrolytic technique for the conjugated 17-ketosteroids of normal male urine with that of HCl has been made.

2. The $BaCl_2$ hydrolysis results in the liberation of 20 to 40 per cent of the total conjugated 17-ketosteroids, of which approximately 50 per cent are of the 3-β-hydroxy configuration.

3. The 3-β-hydroxy 17-ketosteroids thus constitute at least 11 to 16 per cent of the total 17-ketosteroids of normal male urine as indicated by employment of the $BaCl_2$ hydrolysis, as compared to about 4.5 per cent following HCl hydrolysis.

4. The difference in β fractions following $BaCl_2$ and HCl hydrolyses has been shown to be due to the destructive action of HCl on 3-β-hydroxy 17-ketosteroids.

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HEME SYNTHESIS AND RED BLOOD CELL DYNAMICS IN NORMAL HUMANS AND IN SUBJECTS WITH POLYCYTHEMIA VERA, SICKLE-CELL ANEMIA, AND PERNICIOUS ANEMIA*

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Glycine is known to be specifically utilized for the biologic synthesis of the protoporphyrin of hemoglobin (1). The administration of N^{15}-labeled glycine results in the production of red blood cells containing labeled heme. After its formation, the heme as a constituent of hemoglobin remains in the cell until the cell disintegrates. Following the destruction of the red blood cell, little of the heme, if any, is reutilized for new hemoglobin synthesis. By following the isotope concentration in hemin isolated from the red blood cells the length of time that labeled hemoglobin remains in the blood, and thus the survival time of the red blood cells containing the labeled hemoglobin, can be determined. An analysis of such data reveals not only the age distribution at death of the red cell population but also the average life span of the red blood cells (2).

If n_t circulating red cells die at age t, the average life span, $\bar{T}$, of the total circulating red cell population, N, will be given by

$$\bar{T} = \frac{\Sigma n_t \cdot t}{N} \tag{1}$$

In the normal subject it would appear that no significant number of circulating red cells die before they attain the age of 40 days. The death rate of the cells rises to a maximum value at approximately 120 days. This value of 120 days is close to the average life span, $\bar{T}$, of the circulating red blood cells. In the normal subject previously studied by this method the value for $\bar{T}$ was 127 days. This value corresponds well with figures obtained for the human and the dog by other reliable techniques (3-7).

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The average life span, $\bar{T}$, is related to the rate at which red blood cells are delivered to the circulation. This relationship is shown in Equation 2,

$$\bar{T} = \frac{M}{m} \tag{2}$$

in which M is the total mass of circulating red cell hemoglobin and m is the mass of red cell hemoglobin delivered to the circulation per day. This relationship holds only when M is constant; *i.e.*, the rate of synthesis of red cell hemoglobin is equal to the rate of its destruction.

This method of studying red cell dynamics has the unique advantage that it is possible to determine the rate of formation of hemoglobin and of red cells and their pattern of destruction in the same individual in whom the red cells are made and destroyed without altering the usual state of the organism, whether normal or abnormal. Since most disorders of red blood cells result from abnormalities in the synthesis of hemoglobin and red cells or from abnormal destruction of the cells, this method is particularly suited to the study of red blood cell dyscrasias. This report is concerned with studies performed in two normal subjects, male and female, and in subjects with pernicious anemia, sickle-cell anemia, and polycythemia vera.

EXPERIMENTAL

Labeled glycine was prepared from potassium phthalimide and chloroacetic ester (8). Glycine labeled with N^{15} was fed orally over a 48 hour period to each of the subjects. The glycine was fed hourly in equal doses except for triple doses at 12 midnight and 3 a.m., with no other doses between 12 midnight and 6 a.m. The normal male subject, the patient with pernicious anemia prior to treatment, and the patient with polycythemia vera each received 48 gm. of glycine containing 31.7 atom per cent excess N^{15}. The normal female subject received 41.2 gm. of glycine labeled with 30.8 atom per cent excess N^{15}. The patient with pernicious anemia 14 months after the start of liver therapy received 36.2 gm. of 31.7 per cent N^{15} glycine.

20 to 30 ml. of venous blood were drawn at frequent intervals during the course of the experiments and hemin was isolated by the usual procedure (9). In the case of the subject with polycythemia vera, 30 to 50 ml. samples were drawn.

Plasma volume was determined with the blue dye T-1824 and the single 10 minute point technique (10).

Normal Subjects—A 23 year-old white male medical student and a 24 year-old white female medical student served as subjects. Both had normal hematologic findings and had had no serious illnesses in the past.

There was no history of blood dyscrasia in their families. During the course of these experiments, both subjects remained in good health and there was no significant change in hematologic findings.

Pernicious Anemia[1]—This subject was a 51 year-old Negro male with a 19 months history of gradually progressive weakness and weight loss of 25 pounds. Physical and laboratory examinations on admission to the Presbyterian Hospital in November, 1946, revealed a macrocytic anemia and gastric achlorhydria. X-ray examination of the gastrointestinal tract was negative. There was no jaundice or any evidence of combined degeneration of the spinal cord. Hemoglobin, 8.8 gm. per cent (Sahli); red blood cells, 2.0 million per c.mm.; white blood cells, 4800 per c.mm.; neutrophils 54 per cent (0-0-54), eosinophils 2 per cent, lymphocytes 38 per cent, monocytes 6 per cent; platelets 112,000; reticulocytes 0.9 per cent; hematocrit 31 per cent; marked macrocytosis of red blood cells, mean cell diameter 10.2 μ, mean cell volume 155 cu.μ, mean cell hemoglobin 46.5 $\mu\mu$gm. (46.5×10^{-12} gm.), mean cell hemoglobin concentration 27 per cent. A sealed wet smear was negative for sickling after 24 hours. Analysis of gastric juice 30 and 45 minutes after the subcutaneous injection of 1.0 mg. of histamine phosphate revealed no free acid and only 3 units of combined acid. Serum bilirubin 0.8 mg. per cent (indirect).

Polycythemia Vera[2]—This subject was a 58 year-old white housewife with the characteristic physical and laboratory findings of polycythemia vera. Physical and laboratory findings revealed no significant pulmonary or cardiac disease. Laboratory findings included a marked increase in hemoglobin and red blood cell counts, and a mild leucocytosis. Hemoglobin 22.0 gm. per cent (Sahli); red blood cells, 7.2 million per c.mm., with normal differential count; platelets 240,000 per c.mm.; reticulocytes 2 per cent; hematocrit 75 per cent; plasma volume 2690 ml.; arterial hemoglobin oxygen saturation at rest 90 per cent; 1 minute after exercise 92 per cent.

Sickle-Cell Anemia[3]—A 26 year-old Negro male with a history of frequent joint pains since childhood, the development of leg ulcers after minor trauma, frequent recurrent episodes of scleral icterus, and several crises with abdominal pain or generalized aching. The diagnosis of sickle-cell anemia was established on the basis of characteristic hematologic findings. On admission to the Presbyterian Hospital in July, 1947, for the purpose of this study, physical and laboratory findings revealed the following: hemoglobin 10.8 gm. per cent (Sahli); red blood cells 3.8 million per c.mm.; white blood cells 2400 per c.mm.; neutrophils 58 per cent (0-1-57), lymphocytes 33 per cent, monocytes 8 per cent, eosinophils 1 per cent; platelets 282,000 per c.mm.; reticulocytes 10.2 per cent; hematocrit 31 per cent; mean cell volume 82 cu.μ, mean cell hemoglobin 28 μμgm., mean cell hemoglobin concentration 25 per cent. 100 per cent of the red blood cells were sickle-shaped after 24 hours in the sealed wet preparation.

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[1] P. H. 848800.

[2] P. H. 687458.

[3] P. H. 848837.

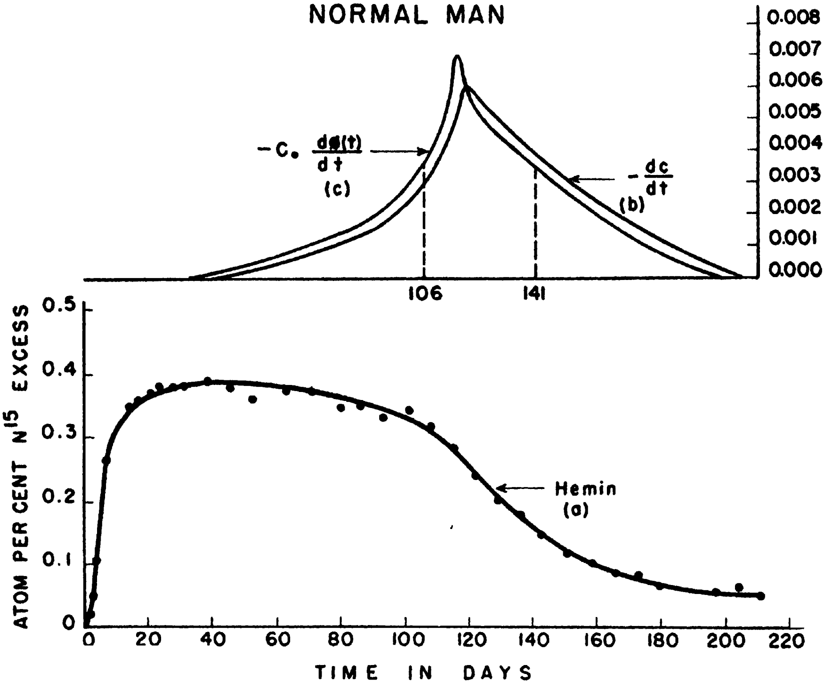

FIG. 1. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days. $C_0 = 0.38$; $\lambda = 0.15$ day^{-1}. The meaning of these symbols in the mathematical treatment of the data has been reported (2).

RESULTS AND DISCUSSION

Normal Subjects—In Figs. 1 and 2 the N^{15} concentrations in hemin following the start of feeding labeled glycine to the two normal subjects are presented. These curves are similar to the one reported earlier (2). The isotope concentration in the hemin rises to a maximum value on about the 30th day; a decline along an S-shaped curve begins between approximately the 40th and 60th days. If, as previously (2), the function $\phi(t)$ is defined as the probability that a red cell will survive in the circulation for a time greater than t, then $-d\phi(t)/dt$ represents the changing probability of survival of a cell population to age t and consequently mirrors the rate of destruction of these cells. In a stationary population $-d\phi(t)/dt$ also represents the age distribution of the cells at death. In Figs. 1 and 2, Curves *a* represent the isotope concentration, *C*, in the hemin throughout the course of the experiment, Curves *b* give the values of $-dC/dt$, and Curves *c* the values of $-C_0(d\phi(t)/dt)$. Curves *c* permit the evaluation of the average life span of the red cells, despite the fact that they have not all been made at one instant.

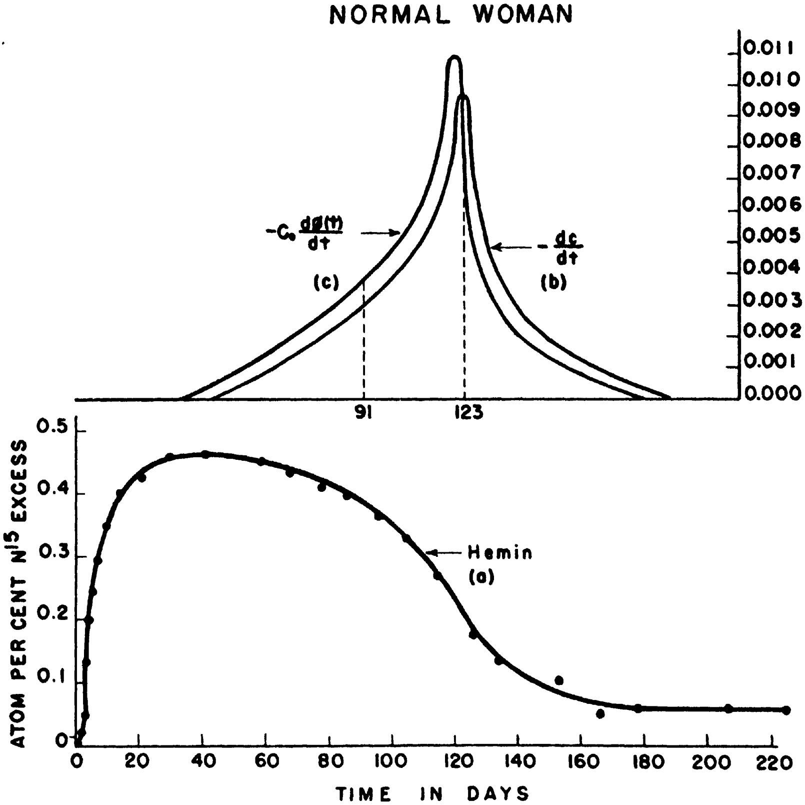

FIG. 2. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days. $C_0 = 0.46$; $\lambda = 0.15\ \text{day}^{-1}$.

In the two normal subjects, Curves *c* attain their maximum value at 117 days (Fig. 1) and 120 days (Fig. 2). These values represent the time when the destruction of the labeled cells is most marked. Inasmuch as the curves are not symmetrical about their maximum ordinates, these values merely approximate the average life span of the red cells. It is possible, however, to determine the average red cell life span more precisely.

From the definition of $\phi(t)$ it is clear that $-N(d\phi(t)/dt)dt$ is the number of cells of age t to $t + dt$ which die in the interval dt. This is equal to n_t. Substitution in Equation 1 yields

$$\bar{T} = \int_0^\infty - t \frac{d\phi(t)}{dt} dt \tag{3}$$

Graphical evaluation of this integral yields values for the average life span of the red cells of 120 and 109 days for the normal male and female subjects, respectively. These values correspond closely to the value of 127 days obtained by this method (2) and to values obtained by the modified Ashby (4, 5) technique in humans.

It is clear that, in the normal human, the red blood cells are destroyed as a function of their age. Despite the rather wide range of red cell survival times (see Curves *c*, Figs. 1 and 2), the time span which encompasses the ages at death of half (the second and third quarters) of the cell population is relatively short. In the male subject this time span is 35 days (106 to 141 days), in the female 32 days (91 to 123 days).

The value of approximately 120 days corresponds to the production and destruction of approximately 0.83 per cent of the red cells per day. The absolute rate, in gm. per day, of production of circulating red cell hemoglobin can be calculated from Equation 2. The total mass of circulating red cell hemoglobin may be calculated from the values for the whole blood volume and the hemoglobin concentration in the blood. The determination of the whole blood volume may be performed by a variety of techniques, of which the use of T-1824 and the use of radioiron-tagged red cells have been most prominent. There is fairly general agreement (11) that the normal human adult male has a plasma volume of approximately 45 ml. per kilo of body weight, the female about 43 ml. per kilo of body weight. There is much less agreement concerning the total red cell volume. Gibson *et al.* (12) claim that the true red cell volume as determined by radioiron-tagged cells is about 15 per cent lower than that determined by T-1824. Values for the red cell volume which are consistent with most of the studies, in which T-1824 or cells tagged with radioiron or radiophosphorus have been used, are about 30 ml. per kilo of body weight for the normal man and about 25 ml. per kilo of body weight for the normal woman (11, 13). The hemoglobin concentration in the peripheral blood is, on the average, 16 and 14 gm. per 100 ml. of whole blood for the normal man and normal woman, respectively (14). Accordingly, there are about 12 and 9.5 gm. of circulating red cell hemoglobin per kilo of body weight in the normal male and female, respectively. With an average red cell life span of 120 and 109 days in our two normal subjects, one can calculate from Equation 2 that the rate of production of circulating red cell hemoglobin in normal man is about 0.10 gm. of hemoglobin per kilo of body weight per day, and in the normal woman about 0.087 gm. of hemoglobin per kilo of body weight per day. With a mean corpuscular hemoglobin value of 29 $\mu\mu$gm. (2.9×10^{-11} gm.) (14) these rates of hemoglobin production are equivalent to the production of 3.45×10^9 red blood cells per kilo of body weight per day for normal man and 3.00×10^9 red blood cells per kilo of body weight per day for the normal female. These values will serve as a basis for comparison of the rates of production of red cells and hemoglobin in the patients with polycythemia vera, sickle-cell anemia, and pernicious anemia.

Polycythemia Vera—Fig. 3 describes the isotope concentration in hemin, the hemoglobin concentration, and red blood cell counts, and indicates when phlebotomies were performed during the course of the experiment. The withdrawal of large amounts of blood produced a lowering of red blood cell and hemoglobin values. The shape of the N^{15} concentration curve in hemin, however, was not affected by the phlebotomies, because the blood withdrawn was a representative sample of the blood in circulation at the time, and the phlebotomies produced no significant change in the generative activity of red blood cells as indicated by reticulocyte counts, which never rose above 3 per cent.

The shape of the curve of N^{15} concentration in hemin is nearly identical with that of the normal curves. The red blood cells are destroyed, as in the normal, as a function of their age, and their average life span calculated in the manner previously described is 131 days. This value of T is close to those values found in normal subjects and is probably within the normal limits. The time span which encompasses the ages at death of half (the second and third quarters) of the cell population is 34 days (116 to 150 days). As can be seen from Equation 2, a normal life span with an abnormally large circulating red cell hemoglobin mass, M, must be associated with an elevated rate of hemoglobin synthesis, m. The plasma volume for this subject at the start of the experiment was 2690 ml. With a venous hematocrit of 75 per cent, the red cell volume would be 7740 ml. But on applying a correction of about 15 per cent (12), the red cell volume is 6580 ml. The total blood volume, then, is 9270 ml. With a hemoglobin concentration of 22 gm. per 100 ml. of whole blood, the total circulating red cell hemoglobin is 2039 gm., or 29.5 gm. of hemoglobin per kilo of body weight (the subject weighed 69 kilos). Since the average life span of the red cells in this subject is 131 days, the rate of hemoglobin production is 0.225 gm. per kilo of body weight per day. With a mean corpuscular hemoglobin content of 3.0×10^{-11} gm., the rate of red cell production is 7.50×10^9 red cells per kilo of body weight per day. These rates of hemoglobin and red cell production are about

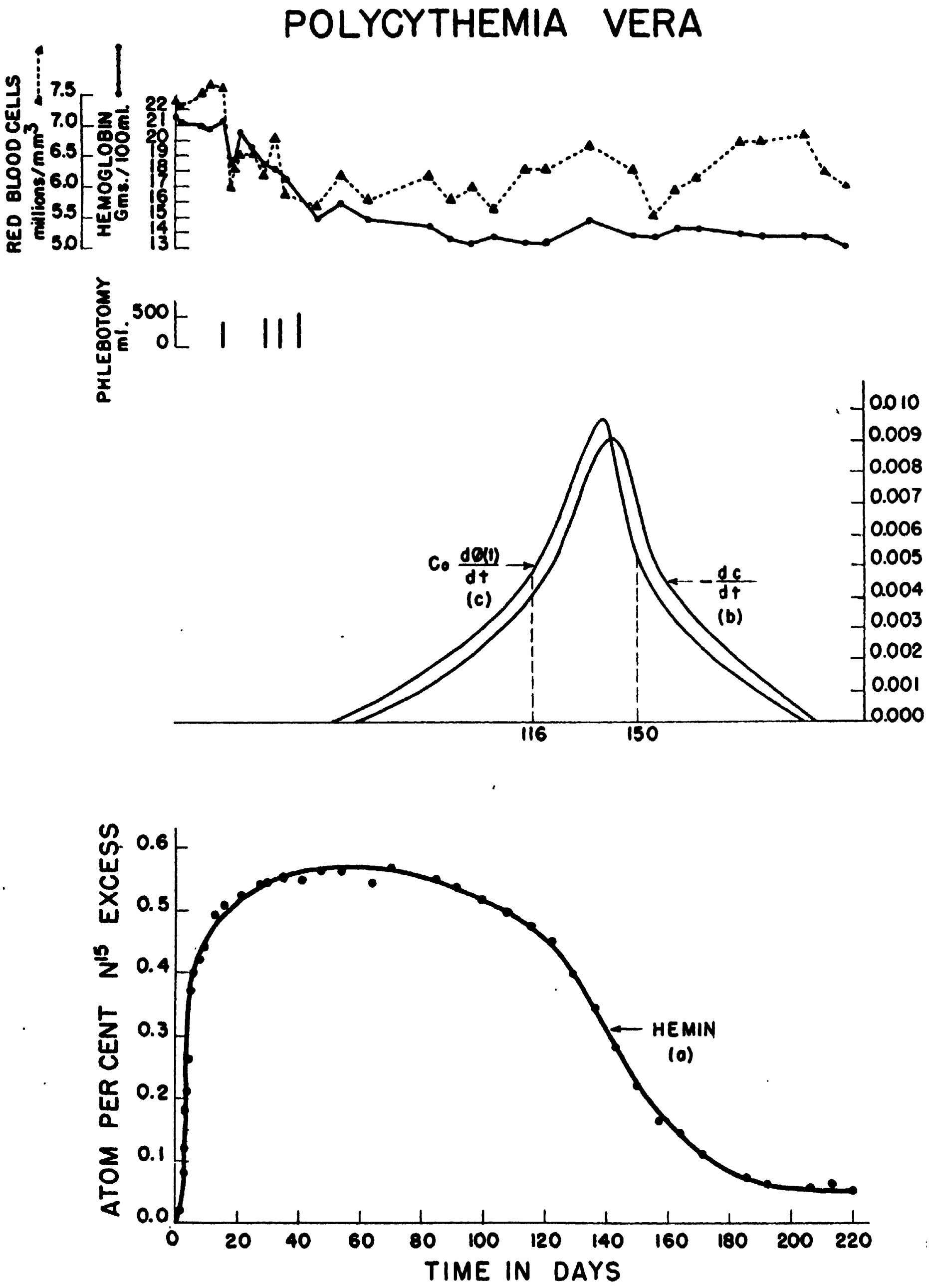

FIG. 3. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days. $C_0 = 0.56$; $\lambda = 0.17\ \text{day}^{-1}$.

2½ times the rate in the normal female. It is to be noted that these values are based on the period during which the labeled red cells were produced and, therefore, are not significantly affected by the therapeutic phlebotomies which, for the most part, were carried out later. The blood withdrawn for hemin N^{15} analyses during the period when the great bulk of labeled cells was formed constitutes a small fraction of the total blood volume and does not affect the general conclusion.

Polycythemia vera, as exemplified by this patient, is characterized by an abnormally high rate of hematopoiesis and a normal red cell life span.

These data throw light on the mechanism of the development of polycythemia vera. Theoretically, two factors, singly or in combination, might produce the marked increase in the total red cell mass which is characteristic of this disease. These are (1) an increased rate of hemoglobin and red blood cell synthesis and (2) prolonged life of the red blood cells. In the normal individual in the steady state, the rate of hemoglobin synthesis equals the rate of hemoglobin degradation, and the average life span of the erythrocyte is the reciprocal of the fraction of the total circulating red cell mass which is synthesized and degraded daily. Thus in the normal individual 0.83 per cent of the total red cell mass is synthesized and degraded daily, and the average life span of the erythrocytes is the reciprocal of 0.0083, or 120 days. The total red cell mass will increase whenever the rate of red cell synthesis is faster than the rate of degradation and will continue to increase until the degradative rate again equals the synthetic rate. A new steady state will then ensue. The finding of a normal red cell life span in this subject at a fully developed stage of the disease is conclusive proof for the existence of a functional hyperactivity of the blood-forming apparatus in the maintenance of the polycythemic state. It seems probable that the development of the polycythemia earlier in the disease is similarly characterized by an increase in hematopoietic activity with the maintenance of a normal erythrocyte life span. The existence of hematopoietic hyperactivity, at least in the maintenance of the polycythemic state, is consistent with the usual findings in polycythemia vera of hyperplasia of all bone marrow elements and evidence in the peripheral blood of increased bone marrow activity (polychromatophilia and basophilic stippling of erythrocytes, and leucocytosis with an increase in immature cells of the myeloid series). The fundamental cause of this functional hyperactivity, however, remains unknown.

A variety of etiologic theories has been proposed, but conclusive evidence in support of any of them is lacking. These theories have been reviewed by Harrop and Wintrobe (15). One of the theories is that of Minot and Buckman (16) who regard polycythemia vera as a form of neoplasm. The persistent bone marrow hyperplasia involving all marrow elements, the development of leucemia in some cases of erythremia, and the development of erythremia in some cases of leucemia suggest that this is a neoplastic process. If polycythemia vera is indeed a benign neoplasm, then at least this neoplastic process is associated with an increase, above normal, of the synthetic activity of the hematopoietic system.

The maintenance of the polycythemic state in the presence of a normal erythrocyte life span requires that the amount of hemoglobin degraded, as well as the amount of hemoglobin synthesized, be increased. However, studies of the excretion of bile pigment in polycythemia vera, which are critically discussed by Watson (17), have revealed fecal urobilinogen values that are much lower than would be anticipated on the basis of the increased amounts of hemoglobin that are degraded. This discrepancy raises questions concerning the fate of the pigment that are pertinent to hemoglobin metabolism in general and warrant further investigation.

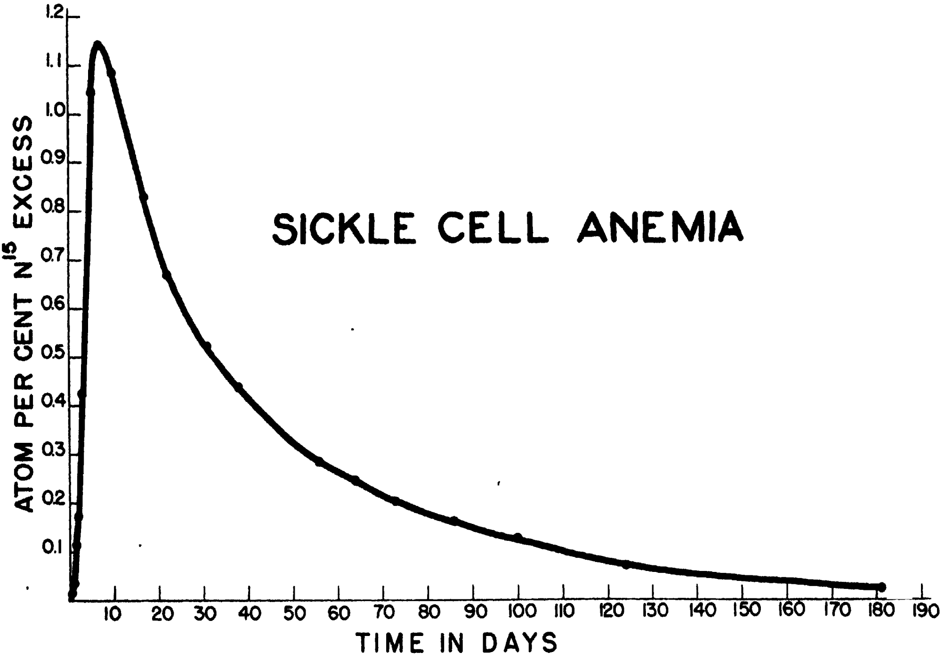

FIG. 4. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days

Sickle-Cell Anemia—The curve of N^{15} concentration in hemin during the course of the experiment is shown in Fig. 4. During the period of the study, the patient suffered no "crises" and the red blood cell, hemoglobin, and hematocrit values remained essentially the same as at the start of the experiment.

The isotope concentration in the hemin rose rapidly to a peak on the 7th day following the start of the feeding of isotopic glycine and immediately began to fall. A direct plot of the isotope concentration in the hemin, C, after the 7th day in a semilogarithmic coordinate system (log C *versus* time) gives a straight line through the major portion of the curve. Theory suggests that a small quantity, Δ, should be subtracted from each value of the isotope concentration of the hemin, since this value is, at each instant, approaching not zero but a finite value representing the isotope concentration of the subject. Δ is not independent of time. However, it varies but slowly with time, approaching zero as a limit. Its value during the course of the last hundred days of this experiment must have been about 0.020 atom per cent excess. We have, therefore, plotted the logarithm of (C – 0.020) against time (see Fig. 5). After the 30th day the data fit a straight line whose equation is

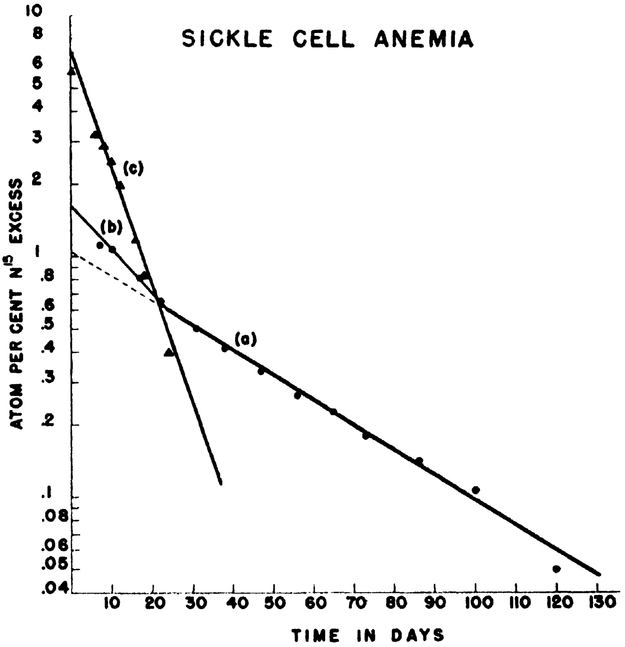

FIG. 5. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days, plotted semilogarithmically. Curve *a* describes N^{15} concentrations in hemin from the 22nd to the 120th day; Curve *b* describes the N^{15} concentrations in hemin prior to the 22nd day; Curve *c* represents a plot of the deviation of Curve *b* from the extrapolation (dotted line) of Curve *a*. The ordinate values of Curve *c* are one-tenth the plotted values.

$$C = 1.02e^{-0.0238t} + 0.020 \tag{4}$$

If we assume that the bulk of the N^{15}-labeled red cells has been formed in a short time interval, then to a first approximation ϕ is given by

$$\phi = e^{-0.0238t} \tag{5}$$

The average time the red cells spend in the circulation, $\bar{T}$, is given by Equation 3. Differentiating Equation 5 and substituting in Equation 3 give

$$\bar{T} = \int_0^\infty - 0.0238te^{-0.0238t}\, dt \tag{6}$$

On integration,

$$\bar{T} = \left[- \frac{e^{-0.0238t}}{0.0238} (1 + 0.0238t) \right]_0^\infty \tag{7}$$

Therefore

$$\bar{T} = \frac{1}{0.0238} = 42 \text{ days}$$

Since $\phi(t)$ is by definition the fraction of a stationary population of cells which are born at a particular moment and will survive to age t, $-(d\phi(t)/dt)$ is the fraction of the population which will die in the interval t to $t + dt$ and $-(1/\phi)(d\phi(t)/dt)$ or $-(d \ln \phi(t)/dt)$ is the death rate of the population of age t. In this case, when $\phi(t) = e^{-0.0238t}$, the death rate is independent of age for $-(d \ln \phi(t)/dt) = 0.0238$. This means that heme is removed from the circulation at a rate which is independent of the age of the heme at the time of its degradation. Such a curve reflects the random occurrence of a single event or of a complex of events which leads to the death of the cell once the initial event has occurred.

This curve could result from (1) a random destruction of the red blood cells and a consequent loss of labeled heme from the circulating blood; or (2) a random degradation and synthesis of heme in circulating red blood cells which are morphologically intact; or (3) random synthesis and degradation of heme in red blood cells which are themselves undergoing random destruction. In the light of our findings in normal subjects which demonstrate that hemoglobin in circulating red blood cells is not in the dynamic state, the second, and, consequently, the third of these possibilities would appear most unlikely. Recent studies (18), however, make it difficult to rule out completely the possibility that some synthesis of heme in the circulating red cells of patients with sickle-cell anemia may occur.

When the whole blood of patients with sickle-cell anemia was incubated with N^{15}-labeled glycine, the hemin isolated from the red cells was found to contain significant concentrations of N^{15} (18). This indicated that heme was synthesized from glycine *in vitro*. Similar significant concentrations were not obtained with the blood of normal subjects or of patients with sickle-cell trait. The synthesis of heme *in vitro* in the blood of patients with sickle-cell anemia occurs at the rate of 0.1 to 0.2 per cent of the red cell heme in 24 hours. If all the hemoglobin in the circulating red blood cells of sickle-cell anemia subjects were synthesized in the peripheral blood at the same rate as in the *in vitro* experiments, the average survival time of the labeled hemoglobin in the circulation would be of the order of 500 to 1000 days. Actually the average survival time, as determined in our subject, is about 40 days.

The major part of the disappearance of heme described in Fig. 4 must be due to a random destruction of cells in sickle-cell anemia. Studies with the Ashby technique (19) are in accord with this view. Any random synthesis of heme that may occur in the peripheral blood of these patients can play only a minor part in the hemoglobin turnover in this disease.

Since the red cells of sickle-cell anemia are destroyed in an indiscriminate fashion rather than as a function of their age, their survival is better designated in terms of their half life time $t_{\frac{1}{2}}$. The half life time of these cells in the circulation is given by the expression

$$t_{\frac{1}{2}} = \bar{T} \times \ln 2 \tag{8}$$

$t_{\frac{1}{2}}$ is therefore equal to 29 days.

It is to be noted that the curve representing Equation 5 consistently lies below the experimental points from the 6th to the 22nd day. *A priori* the contrary would be expected, since during this period some labeled heme is being delivered to the circulation and would tend to result in low values of (d ln C/dt) rather than high ones. When the logarithms of the deviations of the observed data (Curve *b*) from the extrapolation of the major portion (Curve *a*) of the data are plotted against time, Curve *c*, Fig. 5, is obtained. $t_{\frac{1}{2}}$ for this curve is approximately 7 days. This suggests the possibility that there may exist a small fraction of the total red cell population which has a $t_{\frac{1}{2}}$ of approximately 7 days. This value of $t_{\frac{1}{2}}$ is a maximum value, since, as we have indicated above, the accession to the circulation of newly formed red blood cells containing N^{15} would tend to lower the rate of fall of the N^{15} concentration of the total circulating heme during this period. Further studies in patients with sickle-cell anemia and analysis of N^{15} concentrations in the bile pigment excreted during the course of the experiment may throw light on this problem.

With measurements of the total red cell mass and the mean survival time of the red cells, it is possible to calculate the rate of red blood cell and hemoglobin production in this patient. For the mean survival time we shall employ the value of 42 days, which is representative of the great majority of the red cells in circulation.

The plasma volume as determined by T-1824 was reported to be 5000 ml. With a venous hematocrit of 31 per cent, the red cell volume would be 2160 ml. The 15 per cent correction yields a red cell volume of 1836 ml. With a total blood volume of 6836 ml. and a hemoglobin concentration in the blood of 10.8 gm. per 100 ml. of whole blood, the total circulating red cell hemoglobin is 738 gm., or 11.71 gm. of hemoglobin per kilo of body weight (weight of subject, 63 kilos). Since the mean survival time of the red cells is 42 days, the rate of hemoglobin production is 0.279 gm. of hemoglobin per kilo of body weight per day. With a mean corpuscular hemoglobin value of 2.8×10^{-11} gm., the rate of red cell production is 9.96×10^{9} red blood cells per kilo of body weight per day. These rates of hemoglobin and red cell production are about 2.8 times the rate in normal man. Since the hemoglobin and red blood cell counts remained at the same level during the course of the study, it is safe to assume that the patient was also destroying red blood cells at a rate 2.8 times the normal. If there is a fraction of the total red cell population which has a half life time of about 7 days, then this rate of production and destruction, 2.8 times the normal, is minimal. The very rapid formation of erythrocytes is consistent with the markedly hyperplastic bone marrow and the reticulocytosis in the peripheral blood characteristic of this disease. Similarly, a rapid rate of destruction is consistent with the hyperbilirubinemia and increased fecal urobilinogen excretion.

These findings indicate that there is no deficiency in the ability of the hematopoietic organs to make adequate numbers of red blood cells and adequate amounts of hemoglobin. It is clear, however, that the red blood cells are defective in their capacity to survive for a normal erythrocyte life span. That the defect is intrinsic to the cell and not ascribable to any factor in the plasma was shown by Huck (20) and more recently by the use of the Ashby technique (19, 21, 22). The nature of the defect in the cell is still unknown. It would appear to be a defect in the structure of the red cell membrane. This defect may be associated with the sickling process but cannot be ascribed to this phenomenon alone, inasmuch as the erythrocytes of individuals with sickle-cell trait are not abnormally susceptible to destruction (22).

Pernicious Anemia—At the start of the experiment, this patient had never received any form of therapy for pernicious anemia. The isotope concentrations in hemin, and the hemoglobin, red cell, and reticulocyte values are shown in Fig. 6. The isotope concentration in the hemin rose rapidly and was approaching its maximum value on the 15th day. It was considered unwise to withhold treatment longer, and liver extract in large doses was administered intramuscularly. The dosage of liver extract is shown in Fig. 6. The reticulocyte count rose to a peak of 12.8 per cent on the 7th day after the start of therapy, and there was a satisfactory rise in hemoglobin and red cell values. After the start of liver therapy, a fall in the isotope concentration in the hemin occurred. This fall was expected, because the influx of large numbers of new cells formed when the isotope concentration in the glycine had fallen to a low value should result in the dilution of the average isotope concentration in the hemin of circulating red blood cells. The curve continues to decline, however, when the hemoglobin and red blood cell values have approached normal levels, and a mere dilution effect should be minimal. To differentiate between the effect of the dilution and the actual destruction of the labeled red cells, we have calculated total heme N^{15} in circulation during the course of the experiment. Gibson (23) has shown that the

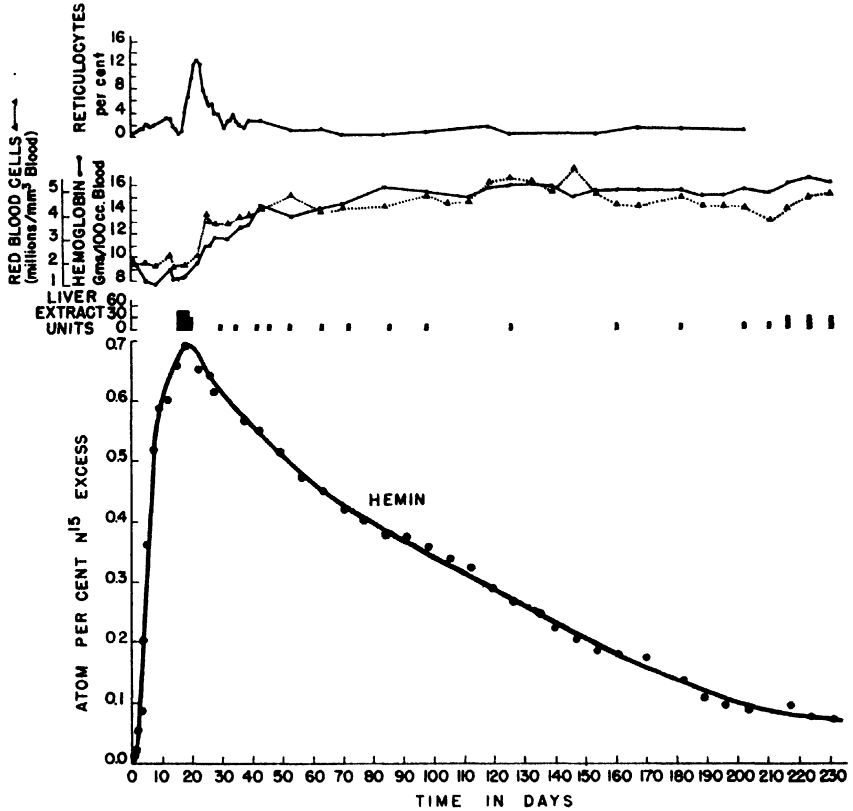

FIG. 6. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days to a subject with pernicious anemia.

hemoglobin concentration in the peripheral blood is a good index of the total hemoglobin in circulation before and after the start of liver therapy in pernicious anemia. By multiplying the hemoglobin concentrations in the peripheral blood by the isotope concentrations in the hemin during the course of the experiment, a curve representing the changes in the total amount of N^{15} in the heme of circulating red blood cells is obtained (Fig. 7).

As in Fig. 6, the curve rises rapidly and is approaching its maximum value on the 15th day. With the administration of liver extract there is a further rise in the total heme N^{15}. This additional rise is due to the influx of many new cells. Although the isotope concentration in the heme of these newly formed cells is relatively low, the large number of these cells newly added to the circulation represents a considerable increment in the total heme N^{15}. Within 2 weeks after the start of liver therapy the peak of the total heme N^{15} curve is reached. If the cells of untreated pernicious anemia enjoyed a normal life span, the curve would have maintained a plateau until about the 40th to 60th day and would then have begun to decline. The curve declines, however, in linear fashion almost immediately after reaching its peak. The linear decline indicates that many but not all of the cells are being destroyed in indiscriminate fashion. If all the cells were destroyed in an indiscriminate manner, the decline of the curve would have been exponential in character. The linear decline suggests that the cell population is mixed, with many of the cells destroyed indiscriminately and others as a function of their age.

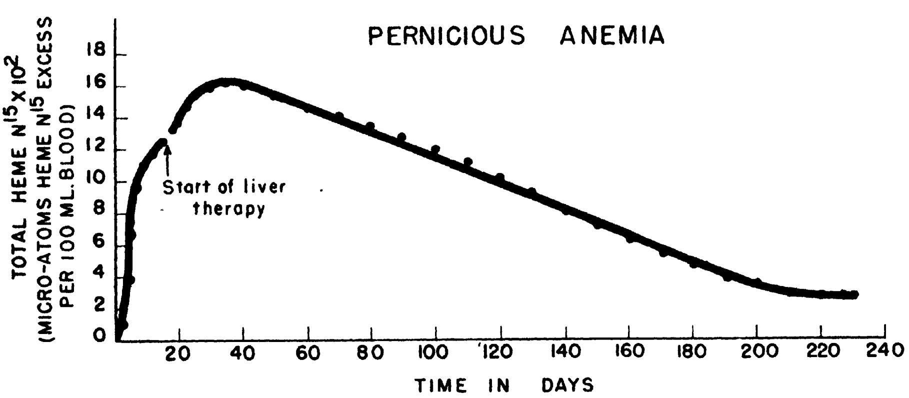

FIG. 7. Total heme N^{15} after feeding N^{15}-labeled glycine for 2 days

The average survival time of the cells of this mixed population (cells formed prior to and after the start of liver therapy) can be estimated from the declining portion of the curve. Approximately three-fourths of the labeled heme was formed prior to the start of liver therapy. It is reasonable to assume that the survival of the cells containing this portion of the labeled heme is not influenced by liver therapy administered after formation and release of these cells into the circulation. The decline in the total hemin N^{15} (Fig. 7) must therefore reflect the disappearance of these labeled cells from the circulation. The time required for the total heme N^{15} to decline from any given value on the declining portion of the curve to one-half that value is about 90 days. This is the half life time for the disappearance of the labeled heme and consequently of the cells containing the labeled heme. As the declining curve is for the most part linear, the half life time and average survival time are equal.

Inasmuch as therapy supervened during the course of the experiment, this value represents the average survival time of the mixed red cell population; *i.e.*, cells formed prior to and after the start of liver therapy. An approximate value for the average survival time of the cells formed before the start of treatment can be obtained.

Fig. 7 represents the sum of the hemin N^{15} of the cells formed before and after the start of liver therapy. It is reasonable to assume that the average survival time of the cells formed after the start of liver therapy is at least as long as the survival time of the cells formed prior to liver therapy. If the survival times of these two groups of cells are equal, the untreated cells will have an average survival time of approximately 90 days; *i.e.*, the same as the survival time of the mixed population. This value represents an upper limiting value for the average survival time of the untreated cells. To establish a lower limiting value, let us assume that the cells formed after the start of treatment have an infinite life span. Inasmuch as these cells contain heme N^{15} representing approximately one-fifth of the total heme N^{15}, were they to have an infinite life span they would account for a lengthening of the mean survival time of the mixed population of about 20 per cent. The survival time of the cells formed prior to treatment would then be approximately 72 days; *i.e.*, about 20 per cent less than that of the mixed population. This represents the lower limiting value. In the fully treated subject with pernicious anemia the average life span of the cells is normal (see below). Following the start of treatment, however, there is most likely an interval prior to the formation of thoroughly normal cells during which cells of varying abnormality are produced. Accordingly, a value closer to the upper limiting value than to the lower will closely approximate the real value for the mean survival of cells of the untreated state. For purposes of further calculation we may choose a value of 85 days. In two cases of untreated pernicious anemia studied by means of the Ashby technique, Loutit (24) found that the red cells survived for 20 and 60 days. Such variations, however, are to be expected, for the severity of the disease and the corresponding abnormalities of the red cells vary widely among patients.

The rate at which newly formed red blood cells and hemoglobin were released into the circulation prior to liver therapy can be calculated by Equation 2. Before the start of treatment with liver extract, the hemoglobin concentration in the peripheral blood was 8.8 gm. per 100 ml. of whole blood, 55 per cent of the average value for the normal adult male. Inasmuch as the hemoglobin concentration in the peripheral blood provides an index of total circulating red cell hemoglobin (23), one may estimate that this patient had approximately 6.6 gm. of hemoglobin per kilo of body weight (55 per cent of 12.0 gm. of hemoglobin per kilo). The rate of hemoglobin production, based on a mean red cell survival time of 85 days, is 0.0786 gm. of hemoglobin per kilo of body weight per day. This value is 78 per cent of the normal rate. With a mean corpuscular hemoglobin content of 4.65×10^{-11} gm., the rate at which red cells are released into the circulation is 1.67×10^{9} cells per kilo of body weight per day. This is only 48 per cent of the normal rate.

The findings of a diminished production of red blood cells capable of reaching the peripheral blood and of a diminished average survival of the red cells in circulation are consistent with the view that the red blood cell of untreated pernicious anemia is intrinsically defective. The absence of an abnormal hemolytic factor in the plasma is suggested by early studies with the Ashby technique (25, 26), and is demonstrated by recent studies with the improved technique in which normal cells transfused to recipients with pernicious anemia enjoyed normal survival (4, 24).

The production of circulating red cell hemoglobin is somewhat diminished. It remains to be determined, however, whether this represents a diminished capacity to synthesize normal hemoglobin or whether it is merely a reflection of the fate of the abnormal red blood cell. But even a normal rate of circulating red cell hemoglobin production and destruction falls far short of providing an adequate explanation for the very large amounts of bile pigment produced in this disease. This discrepancy is explained by the finding in this patient that a very large portion of the stercobilin in the feces apparently was derived from a source other than the hemoglobin of circulating red blood cells (27). These studies, to be reported later, suggest that this portion of bile pigment is derived from one or more of the following sources: (1) hemoglobin of red blood cells which are destroyed shortly after reaching the peripheral blood or which never reach it and are destroyed in the bone marrow; or (2), porphyrins which are not utilized for hemoglobin production; or (3), direct synthesis of bile pigment via a pathway which does not involve degradation of a porphyrin ring.

After treatment with concentrated liver extract for 1 year, the patient was studied again to determine the effects of the treatment on hemoglobin metabolism and red blood cell dynamics. Despite the treatment with concentrated liver extract in a dosage which averaged slightly more than 15 units per week over the 12 months period, a very slight increase in mean cell size persisted: hemoglobin 15.0 gm. per 100 ml.; red blood cells, 4.7 million per c.mm.; hematocrit 46 per cent; mean corpuscular volume 98 cu.μ; mean corpuscular hemoglobin 33 $\mu\mu$gm.; mean corpuscular hemoglobin concentration 33 per cent. To determine whether increased dosage and a cruder extract would reduce the mean cell size, 30 units of concentrated liver extract per week and 60 ml. of Valentine's oral liver extract daily were administered. After 10 weeks of this schedule, the hematologic picture remained essentially unchanged. Examination of the bone marrow revealed no abnormality. To repeat the study, 36.2 gm. of glycine labeled with 31.7 atom per cent excess N^{15} were administered over a 48 hour period. The results of this study are plotted in Fig. 8.

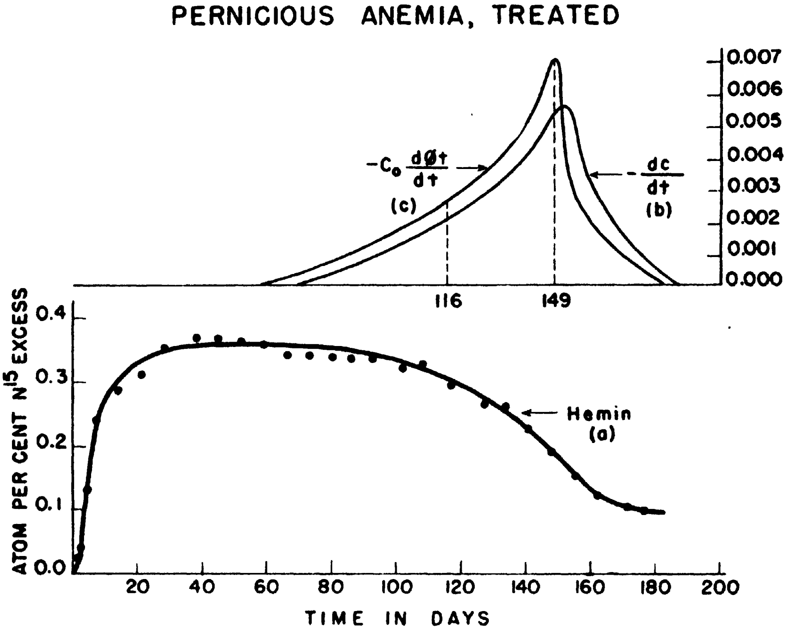

FIG. 8. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days. $C_0 = 0.37$; $\lambda = 0.11$ day^{-1}.

The shape of the curve of isotope concentration in the hemin and the death rate curve, $(d\phi(t)/dt)$, are normal. The red blood cells are destroyed as a function of their age and not in indiscriminate fashion. Half of the red cells (the second and third quarters) die within a 33 day period, 116 to 149 days. The average life span is 129 days, a value very close to the values in the two normal male subjects.

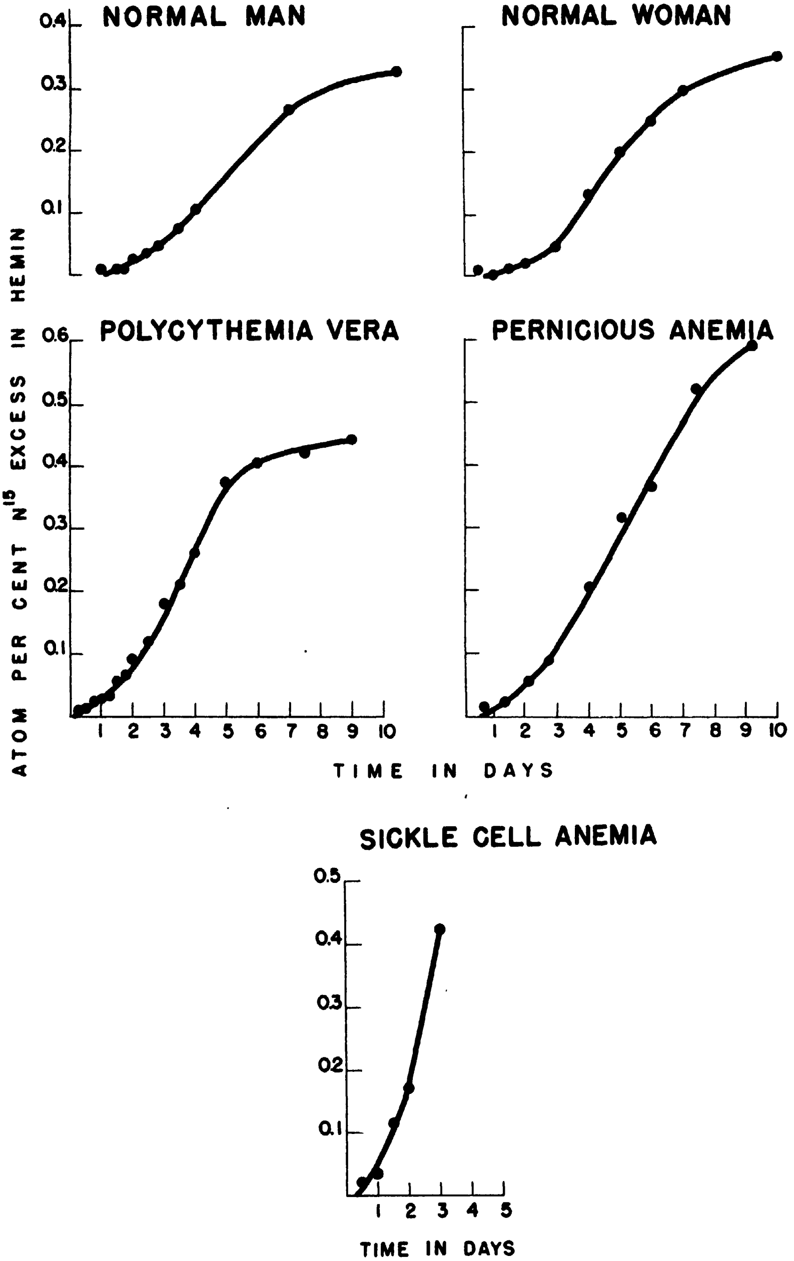

FIG. 9. N^{15} concentration in hemin after feeding N^{15}-labeled glycine for 2 days

Hemoglobin Synthesis and Release of Erythrocytes into Circulating Blood—The concentrations of N^{15} in the hemin during the early part of the experiments in the normal subjects and in the subjects with pernicious anemia (prior to treatment), sickle-cell anemia, and polycythemia vera are shown in Fig. 9. It is noteworthy that red blood cells containing labeled hemoglobin appear in the peripheral blood within several hours after the start of feeding labeled glycine. These findings indicate a rapid utilization and conversion of dietary glycine to protoporphyrin. In addition, they suggest that the human red cell is released into the circulation at a time which approximates the completion of hemoglobin deposition in the cell. If the red cell is mature and not reticulated, hemoglobin deposition is probably completed shortly before the cell enters the circulation. The absence of significant synthesis *in vitro* of heme by normal human blood supports this view (18). If, however, the red cell entering the circulation is reticulated, the process of hemoglobinization may not yet be completed and further hemoglobin synthesis may occur. This hypothesis is supported by the finding that normal mammalian (rabbit) reticulocytes can synthesize heme *in vitro*.[4] The absence of significant heme synthesis *in vitro* in blood from some patients with elevated reticulocyte counts (18) suggests that reticulocytes, although morphologically similar, may differ in their functional capacity to synthesize heme.

SUMMARY

1. The average life span of the circulating red blood cell in a normal human adult male has been found to be 120 days, in a normal human adult female 109 days.

2. A subject with polycythemia vera was shown to have a normal red cell life span of 131 days and a normal pattern of red cell destruction, but a rate of red cell and hemoglobin production about 2½ times the normal. The mechanism of the development of polycythemia vera is discussed in the light of these findings.

3. In sickle-cell anemia the red blood cells were shown to be destroyed indiscriminately rather than as a function of their age. Their mean survival time in the subject studied was 42 days, their half life time 29 days. The rates of hemoglobin and red cell formation and destruction were about 2.8 times the rates in normal man.

4. Study of a subject with untreated pernicious anemia disclosed an abnormal pattern of red cell destruction and a mean survival time of approximately 85 days. The rate of production of circulating red cell hemoglobin was found to be about four-fifths the normal, the rate of production of circulating red cells about half the normal rate. Treatment with liver extract resulted in restoration of the pattern of red cell destruction to normal and in a normal red cell life span of 129 days.

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[4] London, I. M., Shemin, D., and Rittenberg, D., unpublished data.

5. The utilization of dietary glycine for the synthesis of the protoporphyrin of hemoglobin is very rapid, and cells containing labeled hemoglobin are released into the circulation within several hours after the start of feeding labeled glycine. The time relationships between hemoglobin deposition in the red cell and release of the red cell into the circulation are discussed.

We are indebted to Dr. Joan Morgenthau, Dr. Lillian Strange, and Dr. Gilbert Gordon for their cooperation, to Miss Martha Yamasaki for her assistance, to Mr. I. Sucher for the isotope analyses, to Miss Florence Schorske for the plasma volume determinations, and to Mrs. Catherine Holavko and Miss Gloria Sabella for hematologic technical assistance.

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LETTERS TO THE EDITORS

INABILITY OF VITAMIN B_{12} TO REPLACE THE DESOXYRIBOSIDE REQUIREMENT OF A LACTOBACILLUS BIFIDUS

Sirs:

A strain of *Lactobacillus bifidus*[1] isolated from a stool of a breast-fed infant could be grown on a chemically defined medium, provided the casein supplement were an enzymatic digest and not an acid hydrolysate. The activity of the digest appears to have been introduced with the enzyme preparation, since a commercial pancreatin, when tested as the supplement to the basal medium containing acid hydrolysate of casein, exhibited high activity. Of a number of compounds tested, desoxyribonucleic acid, thymine desoxyriboside, and guanine desoxyriboside proved active, in accordance with the re-

	Supplement No.	0.02 N NaOH minus blank*
		cc.
(1)	Blank	(1.7)
(2)	50 γ desoxyribonucleic acid	11.3
(3)	5 “ “ “	3.1
(4)	5 “ thymine desoxyriboside†	9.7
(5)	1 “ “ “	5.0
(6)	5 “ guanine “ †	6.5
(7)	1 “ “ “	3.2
(8)	0.001–1.0 γ vitamin B_{12}†	0.0
(9)	100 γ thymine	0.0
(10)	10 mg. ascorbic acid (non-autoclaved)	0.0
(11)	50 γ ribonucleic acid	0.0
(12)	(3) + (11)	3.0
(13)	50 γ pancreatin, VioBin	11.5

* 16 hours anaerobic incubation at 37° in 2 cc. of a medium similar to that of Roberts and Snell (*J. Biol. Chem.*, **163**, 499 (1946)) with acid hydrolysate of casein substituted for tryptic digest and fortified with 0.1 per cent Tween 80 and 3.5 per cent lactose.

† We are indebted to Dr. W. Shive for the thymidine, to Dr. S. Cohen for the guanine desoxyriboside, and to the Merck Laboratories for the vitamin B^{12}.

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[1] On primary isolation this strain exhibited the bifid characteristic but on repeated subculture converted to a rod-like morphology.

cent report[2] that the thymidine growth effect is not specific but dependent on the desoxyriboside structure. In contrast to the behavior of other strains of *Lactobacilli*,[3] crystalline vitamin B_{12} even at high levels could not replace the desoxyribosides for the growth of this strain of *L. bifidus*. Ribonucleic acid, thymine, and ascorbic acid, alone and in combination with vitamin B_{12}, were also inactive. No inhibition of desoxyribonucleic acid was observed when ribonucleic acid was added at a 5 times greater concentration. It has been postulated that vitamin B_{12} functions in the synthesis of the desoxyriboside component of nucleic acid.[2, 3] For our strain of *L. bifidus*, vitamin B_{12} is not the limiting factor. The high potency of pancreatin, equivalent to that of desoxyribonucleic acid, suggests that the crude enzyme preparation contains a factor or factors much more active than the nucleosides. It is not crystalline vitamin B_{12} but may be one of the other six entities[4] demonstrated in samples possessing vitamin B_{12} or animal protein factor activity.

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Paul György

Received for publication, March 7, 1949

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EFFECT OF THE ANIMAL PROTEIN FACTOR ON THE REQUIREMENT FOR METHYLATING COMPOUNDS*

Sirs:

The choline requirement of chicks fed a purified diet has been found to be approximately 0.1 per cent of the diet.[1] With practical diets, however, more rapid growth is frequently obtained when betaine, choline, or other methylating compounds considerably in excess of this requirement are provided. Since such diets, unsupplemented, would not ordinarily be considered deficient in methyl groups, it is of considerable interest to determine the factors which influence the need for biologically available methyl groups.

Supplement	Weight, end of 3 wks. depletion period*	Gain in weight, 4 to 7 wks., inclusive†
	gm.	*gm.*
None	146	248
0.2% betaine·HCl	148	339
0.2% choline·Cl	144	335
0.15% liver paste‡	144	450
0.15% " " + 0.2% betaine·HCl	149	454
0.15% " " + 0.2% choline·Cl	147	445

* During the first 3 weeks all the chicks received the unsupplemented basal diet which had the following percentage composition: yellow corn-meal 46, ground whole wheat 15, pulverized oats 5, soy bean oil meal 30, fish liver oil 0.5, $CaHPO_4$ 2, $CaCO_3$ 1, NaCl 0.48, $MnSO_4 \cdot 4H_2O$ 0.02; plus riboflavin 0.1 gm., niacin 1.0 gm., and calcium pantothenate 0.25 gm. per 100 pounds.

† Nineteen white Leghorn male chicks were used in each lot, and no mortality occurred during the period from 4 to 7 weeks.

‡ Wilson and Company's 95 per cent alcohol-soluble liver extract paste. This amount has been found to supply the chick's requirement for APF.

In a series of experiments dealing with this problem, evidence has been obtained that the need for supplementary methylating compounds is directly influenced by the animal protein factor (APF). The results of one experiment which are typical of those that have been obtained with both normal and partially APF-depleted chicks are presented in the table. A diet complete in all known vitamins and composed of mixed cereals, plant protein, and supplementary minerals and vitamins was significantly

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* Supported in part by a grant from the International Minerals and Chemical Corporation, Chicago, Illinois.

[1] Jukes, T. H., *Proc. Soc. Exp. Biol. and Med.*, **46**, 155 (1941). Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).

improved by additions of betaine or choline. Since betaine was as effective as choline in promoting the growth of chicks on this diet, it is probable that the effect was due to the increased methyl groups provided. However, the addition of 0.15 per cent of liver extract paste to the basal diet was considerably more effective in promoting growth than betaine or choline. Furthermore, when the liver paste was included, no improvement in growth was obtained by adding these methylating compounds. The amount of choline contributed to the diet by the level of liver paste used was negligible (0.0018 per cent). This particular liver paste has been used in our laboratory for a considerable period of time as a source of APF.[2] It contains approximately 17 γ of vitamin B_{12} activity per gm. by assay with *L. leichmannii* (ATCC 4797).

The results obtained in these studies show that the inclusion of a source of APF in the basal diet relieved the need for supplementary methylating compounds under the experimental conditions. This indicates that at least one metabolic function of APF is concerned with transmethylation. It appears probable either (*a*) that transmethylation is more efficient in the presence of an adequate amount of APF or (*b*) that a partial deficiency of APF creates or stimulates metabolic processes which require excess methyl groups. Further work is required to show whether the activity of the liver paste is due to vitamin B_{12} or to some other component of APF.

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[2] Gillis, M. B., Heuser, G. F., and Norris, L. C., *J. Nutr.*, **23**, 153 (1942); and unpublished work.

XANTHINE OXIDASE IN THE LIVERS OF RATS RECEIVING PURIFIED AND STOCK DIETS

Sirs:

It has been reported recently by Keith *et al.*[1] that chick liver xanthine oxidase is inversely related to the folic acid content of the purified diet fed. These investigators also found the average xanthine oxidase activity of livers of chicks fed a *commercial* diet to be less than for chicks fed a *purified* diet plus excess folic acid. Westerfeld and Richert[2] reported an opposite effect with rats fed commercial and purified diets. They found that normal weanling rats which possess about one-half the average xanthine oxidase activity of healthy, mature rats showed no change in this starting level when fed a purified diet for 6 weeks. However, when the weanling rats were placed on a commercial diet, or the purified diet plus raw cream, the xanthine oxidase activity increased to near normal within 2 weeks. This was taken to indicate the existence of a new dietary factor essential for maintenance of normal levels of liver xanthine oxidase.

Xanthine oxidase activity is expressed as c.mm. of oxygen absorbed per hour per gm. of whole tissue. Group I animals were fed the purified diet used by Petering and Delor* from 28 days. Group II received the same purified diet, plus a supplement of 50 γ of folic acid per 100 gm. of diet. Group III rats were fed an adequate stock diet from weaning time. The age of the rats varied from 10 to 40 weeks.

Group I		Group II		Group III	
Rat No.	Activity	Rat No.	Activity	Rat No.	Activity
1	176	1	118	1	593
2	126	2	178	2	408
3	350	3	116	3	468
4	168	4	306	4	367
5	226			5	360
6	294			6	312
7	292			7	360
8	250				
Average....	235		180		410

* Petering, H. F., and Delor, R. A., *Science*, **105**, 547 (1947).

During the course of our studies[3] on the inhibition of xanthine oxidase activity, we have observed the same effect noted by Westerfeld and Richert.

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[3] To be published later.

Our data indicate a somewhat higher xanthine oxidase activity in the livers of rats fed an adequate stock diet than in those fed a purified diet containing 2 per cent sulfasuxidine, either with or without added folic acid. When folic acid was added to the purified diet we found the rat liver to show a small but noticeable decrease in enzyme activity.

The xanthine oxidase activity of the livers was measured by the method of Axelrod and Elvehjem.[4] The data shown in the table seem sufficient to indicate that rat liver xanthine oxidase activity in animals receiving a purified diet is somewhat less than in animals receiving adequate nutrition. Because of the small number of animals studied and the individual variations in activity of the livers, the small decrease in enzyme activity when folic acid was added to the purified diet may not be significant, but it does correspond qualitatively to Keith's findings with chicks.

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[4] Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **140**, 725 (1941).

ENZYMATIC SYNTHESIS OF CITRIC ACID BY CONDENSATION OF ACETATE AND OXALACETATE*

Sirs:

There is evidence that oxidative breakdown of carbohydrate and fatty acids leads to a common acetyl derivative, which through condensation with oxalacetate undergoes complete oxidation via the tricarboxylic cycle.[1] Recent work strongly suggests that coenzyme A (Co A) is involved

The values are measured in micromoles.

	Citrate found					
	Complete	No Co A	No ATP	No Mg^{++}	No OAA	No acetate
With acetate	0.86, 1.26	0.06	0.09	0.57	0	0.06
" acetoacetate	0.39	0.07	0.07		0	

Additions	Citrate found			*d*-Isocitrate found		
	15 min.	30 min.	60 min.	15 min.	30 min.	60 min.
Complete system	0.30	0.45	1.17	<0.005	<0.005	<0.005
cis-Aconitate (1 μM)	0	0	0.17	0.04	0.09	0.22
d-Isocitrate (1 μM)	0	0.05	0.09	0.90	0.82	0.69

1.0 ml. of the complete system contained: enzyme (12 to 15 mg. of protein) in 0.02 M $NaHCO_3$, 0.4 ml.; 0.025 M potassium phosphate buffer, pH 7.0; Co A, 2.25 to 5 units; oxalacetate (OAA), 17 μM; acetate (or acetoacetate), 20 μM; ATP, 3 μM; $MgCl_2$, 4 μM, L-cysteine, 10 μM (no Co A, ATP, OAA, or acetate present in samples with added tricarboxylic acids). Incubated 60 minutes (unless otherwise stated) in 5 per cent CO_2 and 95 per cent N_2 at 25°. Citric acid determined chemically (Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **38**, 426 (1944)); isocitric acid enzymatically (Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948)). We are indebted to Dr. D. Nachmansohn for a generous gift of a Co A preparation made by Parke, Davis and Company. This material was partially purified by precipitation with barium salts and acetone. The same results were obtained with a highly purified sample of Co A kindly supplied by Dr. F. Lipmann.

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* Aided by grants from the United States Public Health Service, the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the Office of Naval Research, and the Rockefeller Foundation.

[1] Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).

in the above condensation[2] and shows that an active acetate can be generated from acetate and ATP.[3, 4]

We find that ammonium sulfate fractions from extracts of acetone-dried pigeon liver, prepared and aged as described by Kaplan and Lipmann,[3, 5] readily form citrate from acetate (or acetoacetate) and oxalacetate in the presence of ATP, Co A, and Mg^{++} (see the table).[6] The aconitase content of some preparations is so low that equilibrium between the three tricarboxylic acids fails to be established. It thus appears that citrate, rather than *cis*-aconitate or isocitrate, is the condensation product. Condensation between an acetyl phosphate[4] and oxalacetate may result in the intermediate formation of citryl phosphate.

Since acetoacetate is less effective than acetate in forming citrate, it may be concluded that the keto acid does not condense with oxalacetate and must first undergo cleavage.

Oxalacetate markedly depresses the synthesis of acetoacetate and the acetylation of sulfanilamide. This may indicate, as suggested by Soodak and Lipmann[3] for the above reactions, that the same acetyl derivative is involved in the three condensations.

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[2] Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **171**, 833 (1947). The rôle of pantothenic acid in pyruvate oxidation was demonstrated by Dorfman, A., Berkman, S., and Koser, S. A. (*J. Biol. Chem.*, **144**, 393 (1942)).

[3] Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948). Soodak, M., and Lipmann, F., *J. Biol. Chem.*, **175**, 999 (1948). Activity of ATP + acetate in biological acetylations (choline) was first demonstrated by Nachmansohn and Machado (*J. Neurophysiol.*, **6**, 397 (1943)).

[4] This may be the ATP-acetate reaction product described by Kaplan and Lipmann (*J. Biol. Chem.*, **176**, 459 (1948)).

[5] Kaplan, N. O., and Lipmann, F., *Federation Proc.*, **6**, 266 (1947).

[6] The presence of cysteine is essential.

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THE SYNTHESIS OF GLUTATHIONE IN CELL-FREE PIGEON LIVER EXTRACTS*

Sirs:

It has been shown previously that isotopic glycine is incorporated into glutathione in rat liver slices[1] and in homogenates of pigeon liver.[2] Further study has shown that glutathione synthesis, as measured by the incorporation of C^{14}-glycine, also proceeds rapidly in the supernatant fluid of pigeon liver homogenates which had been centrifuged at 2000*g* for 10 minutes. Practically no enzyme activity remains in the sediment and addition of heat-inactivated supernatant fluid or of cytochrome *c*, adenosine triphosphate, and succinate to the particulate matter is without effect.

Incorporation of C^{14}-Glycine into Glutathione in Extracts of Acetone-Dried Pigeon Liver

5 gm. of acetone powder from pigeon liver were extracted with 55 ml. of 0.15 M NaCl, 0.02 M $NaHCO_3$, and 0.001 M cysteine. Each flask contained 5 ml. of centrifuged extract, 1.2 ml. of 0.32 M glutamate, 1.2 ml. of 0.16 M C^{14}-glycine (specific activity 100,000 c.p.m.), 0.4 ml. of 0.16 M cysteine, 1 ml. of 0.15 M KCl, 2.5 ml. of 0.1 M phosphate buffer (pH 7.4), and 0.5 ml. of 0.15 M $MgSO_4$. Total volume, 12.2 ml. The solutions were incubated in N_2 at 37° for 1 hour. After incubation, 30 mg. of carrier glutathione were added and glutathione isolated as the cuprous mercaptide (Waelsch, H., and Rittenberg, D., *J. Biol. Chem.*, **139**, 761 (1941)).

Addition	Specific activity in glycine moiety of glutathione
	counts C^{14} per min.
1. ATP 0.001 M	3315
2. None	73
3. ATP 0.001 M, NH_4Cl 0.002 M	2194
4. ATP 0.001 M, glutamate replaced by L-glutamine	705
5. Same as (1), extract heated at 100° for 5 min.	10

Saline-bicarbonate extracts of acetone-dried pigeon liver were found to be as active enzymatically as whole homogenates. The C^{14} content of the glutathione in Experiment 1 corresponds to the synthesis of 1.7 mg. of the tripeptide per gm. of dried liver per hour. The data in the table demonstrate that in such extracts the formation of labeled glutathione requires the presence of adenosine triphosphate. In this system glutamine only partially replaces glutamic acid. Ammonium chloride in relatively low concentrations depresses the level of synthetic activity. These two observations indicate that glutamine is not an intermediate in glutathione synthesis, and therefore that ATP does not exert its effect through the formation of glutamine.[3]

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* Aided in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Bloch, K., and Anker, H. S., *J. Biol. Chem.*, **169**, 765 (1947).

[2] Bloch, K., unpublished work.

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[3] Speck, J. F., *J. Biol. Chem.*, **168**, 403 (1947).

[4] National Cancer Institute Predoctorate Research Fellow.

THE FORMATION OF THE β-CARBON OF SERINE FROM CHOLINE METHYL GROUPS*

Sirs:

In a previous communication[1] we have presented evidence indicating that in the rat glycine is converted to serine via condensation with formate or a formate derivative. Some of this "formate" is formed from the α-carbon of glycine itself.[2]

	COOH*	α*	β*
Serine......................................	5	11	1660

The administered choline contained 1.66 × 10^5 counts per minute per mg. of methyl carbon. 14 per cent of the choline C^{14} was recovered in the respiratory CO_2.

* Counts per minute per mg. of carbon.

In the present investigation we have studied the possibility that "formate" may also be formed from one or more of the choline methyl groups. According to this hypothesis, liver serine isolated after the administration of C^{14}-methyl-labeled choline should contain C^{14} in the β position.

Two male rats weighing a total of 333 gm. were given 5 mM of glycine per 100 gm. by stomach tube and 0.045 mM of C^{14}-methyl-labeled choline per 100 gm. subcutaneously. The latter dose was repeated at 2 and 4 hours following the original injection. After 14 hours the animals were sacrificed. Serine was isolated from the livers and degraded as previously described.[1]

The results of the C^{14} analyses, shown in the table, support the hypothesis that at least one of the methyl groups of choline may be converted to "formate." The serine contained high C^{14} activity, almost all of which was located in the β position.

This experiment indicates that one or more of the choline methyl groups may be converted to formate or a formate derivative and condensed with glycine to form the β-carbon of serine. The *in vivo* oxidation of the methyl

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* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and by support of the Elisabeth Severance Prentiss Foundation.

[1] Sakami, W., *J. Biol. Chem.*, **176**, 995 (1948).

[2] Sakami, W., in press.

group of methionine to CO_2, reported by Mackenzie *et al.*,[3] may occur over this pathway, since this group is transferable to choline.[4]

The author wishes to express his appreciation to Dr. H. G. Wood for his interest in this investigation.

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[3] Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **169**, 757 (1947).

[4] du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941). Keller, E. B., Rachele, J. R., and du Vigneaud, V., *J. Biol. Chem.*, **177**, 733 (1949).

[5] With the technical assistance of Jean Lafaye.

THE ENZYMATIC TRANSFORMATION OF GALACTOSE INTO GLUCOSE DERIVATIVES

Sirs:

Extracts of galactose-fermenting yeasts contain the enzyme galactokinase,[1] which catalyzes a transphosphorylation between adenosine triphosphate and galactose. The reaction product galactose-1-phosphate was known to be transformed by crude extracts[2] probably to glucose-6-phosphate.

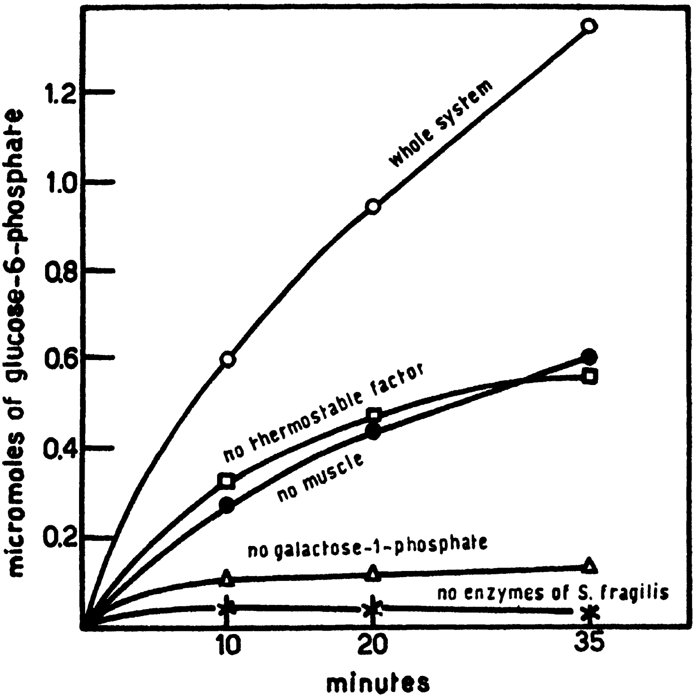

FIG. 1. The transformation of galactose-1-phosphate into glucose-6-phosphate. Whole system, 2 μM of galactose-1-phosphate, 1 μM of $MgSO_4$, 0.03 ml. of partially purified *S. fragilis* enzyme, 0.01 ml. of muscle extract containing phosphoglucomutase, and 0.05 ml. of purified thermostable factor from yeast; total volume, 2.3 ml. The glucose-6-phosphate is measured by its reducing power.[3]

A study of this reaction showed that, when a partially purified enzyme of *Saccharomyces fragilis* was used, two additional factors are necessary for maximum activity (Fig. 1). One is thermolabile and the other thermostable. The thermolabile factor is present in muscle and has been identified with phosphoglucomutase by using this enzyme as purified by Najjar,[4] or yeast extract plus glucose diphosphate.[3, 5] The reaction would be

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[1] Trucco, R. E., Caputto, R., Leloir, L. F., and Mittelman, N., *Arch. Biochem.*, **18**, 137 (1948).

[2] Kosterlitz, H. W., *Biochem. J.*, **33**, 1087 (1939). Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E., and Paladini, A., *Arch. Biochem.*, **18**, 201 (1948).

$$\text{Galactose-1-phosphate} \xrightarrow[(a)]{} \text{glucose-1-phosphate} \xrightarrow[(b)]{} \text{glucose-6-phosphate}$$

In the absence of phosphoglucomutase, glucose-1-phosphate accumulates, as may be ascertained by destroying the *S. fragilis* enzyme by heating, adding phosphoglucomutase, and then measuring the glucose-6-phosphate formed.

The thermostable factor has been found to act in reaction (*a*), and is different from glucose diphosphate, which acts in reaction (*b*). This factor is present in mammalian liver and in commercial yeast. It is hoped that its identification will cast some light on the long sought mechanism of the inversion at C_4 in hexoses.

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[3] Paladini, A. C., Caputto, R., Leloir, L. F., Trucco, R. E., and Cardini, C. E., *Arch. Biochem.*, in press.

[4] Najjar, V. A., *J. Biol. Chem.*, **175**, 281 (1948).

[5] Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A., and Caputto, R., *Arch. Biochem.*, **19**, 339 (1948).

DISTRIBUTION OF LABELED CARBON IN PLANT SUGARS AFTER A SHORT PERIOD OF PHOTOSYNTHESIS IN $C^{14}O_2$[*]

Sirs:

Aronoff *et al.*[1] reported that sugars isolated from barley seedlings which had assimilated $C^{14}O_2$ contained labeled carbon in all positions. Most of the label was in carbons 3 and 4, while carbons 1 and 6 had the least amount of label and positions 2 and 5 were intermediate.

Sugar	Organism	Carbons 1.6	Carbons 2.5	Carbons 3.4
Animal glucose	Rat	3.3	2.0	94.7
1 hr. monosaccharide	Barley	56	28	15
2 " sucrose	"	36	33	31
24" "	Canna	34	33	33

The figures in the table represent the percentage of radiocarbon in the various positions.

It has been found in this laboratory that sugars isolated from plants which have photosynthesized in $C^{14}O_2$ for 1, 2, and 24 hours have radioactive carbon in all positions; however, in the 1 and 2 hour periods of photosynthesis in barley the distribution of the label was opposite to that reported by Aronoff, Barker, and Calvin. The greatest percentage of the label was in positions 1 and 6, while positions 3 and 4 contained the smallest percentage of radioactive carbon.

The sugars were degraded according to the method of Wood *et al.*[2] *Lactobacillus casei* Strong, obtained from the American Type Culture Collection, was used to ferment the sugars to lactic acid. Sucrose was degraded by hydrolysis with invertase, followed by *L. casei* fermentation.

In order to check the procedures used in degradation, glucose obtained by hydrolysis of labeled rat liver glycogen prepared by the method of Zilversmit *et al.*[3] was degraded by the identical procedure used for the plant sugars. The degradation results on this hydrolyzed glycogen agree with those recently reported by Shreeve *et al.*[4]

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[*] This research was carried out at the Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

[1] Aronoff, S., Barker, H. A., and Calvin, M., *J. Biol. Chem.*, **169**, 459 (1947).

[2] Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).

[3] Zilversmit, D. B., Chaikoff, I. L., Feller, D. D., and Masoro, E. J., *J. Biol. Chem.*, **176**, 389 (1948).

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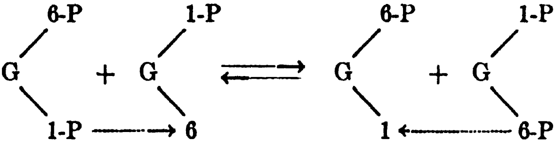
[4] Shreeve, W. W., Feil, G. H., Lorber, V., and Wood, H. G., *J. Biol. Chem.*, **177**, 679 (1949).

THE MECHANISM OF ACTION OF PHOSPHOGLUCOMUTASE AND PHOSPHOGLYCERIC ACID MUTASE

Sirs:

It was shown in a previous note[1] that the conversion of glucose-1-phosphate (G-1-P) to glucose-6-phosphate (G-6-P) by crystalline phosphoglucomutase requires the presence of catalytic amounts of glucose-1,6-diphosphate (G-1,6-P), confirming a scheme proposed by Leloir and coworkers.[2]



When α-G-1-P containing C^{14} and P^{32} was incubated with enzyme and non-radioactive synthetic α-G-1,6-P, the radioactivity of both the carbon and phosphorus became evenly distributed among the three compounds participating in the reaction, in agreement with the above scheme.

In an analogous manner the mutase which catalyzes the reaction, 3-phosphoglyceric acid (3-P-GA) ⇄ 2-phosphoglyceric acid (2-P-GA), is activated by catalytic amounts of 2,3-diphosphoglyceric acid (2,3-P-GA), a compound which Greenwald[3] isolated from red blood corpuscles. This can be demonstrated in dialyzed extracts of previously perfused muscle as well as with a protein fraction which precipitates between 0.4 and 0.5 saturation with ammonium sulfate. In the two experiments shown the conversion of 2-P-GA to 3-P-GA was measured polarimetrically.[4]

The catalytic action of 2,3-P-GA could also be demonstrated in a system consisting of purified enolase and phosphoglyceric mutase. Starting with 3-P-GA, the formation of phosphopyruvate (measured spectrophotometrically at 240 mμ[5]) was accelerated by 2,3-P-GA; likewise, after the enolase equilibrium had been established with 2-P-GA, the disappearance of phosphopyruvate on addition of mutase was accelerated by 2,3-P-GA. In a representative experiment, during 20 minutes of incubation, 0.11 μM of phosphopyruvate per ml. disappeared without and 3.4 μM with the addition of a catalytic amount of 2,3-P-GA; the latter value corresponded to that expected for the enolase-mutase equilibrium.

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[1] Sutherland, E., Posternak, T., and Cori, C. F., *Federation Proc.*, **8**, 258 (1949).

[2] Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A., and Caputto, R., *Arch. Biochem.*, **19**, 339 (1948).

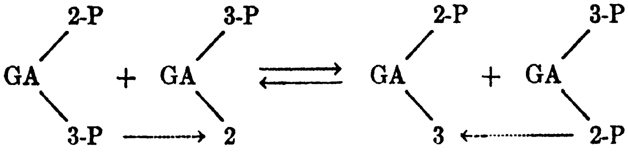
[3] Greenwald, I., *J. Biol. Chem.*, **63**, 339 (1925).

[4] Meyerhof, O., and Schulz, W., *Biochem. Z.*, **297**, 60 (1938).

[5] Warburg, O., *Biochem. Z.*, **310**, 384 (1942).

D(−)-3-P-GA containing P^{32} was incubated with enzyme and non-radioactive 2,3-P-GA. Separation of the components was effected by the differential solubility of their brucine salts in water, 2,3-P-GA being the least soluble. The distribution of P^{32} was in accord with the following equation (which is analogous to that given for phosphoglucomutase).

Ammonium sulfate fraction	Time of incubation (at 30°, pH 7.4)	D(+)-2-P-GA at 0 time (added as DL)	2,3-P-GA added	3-P-GA formed
	min.	*μM per ml.*	*μM per ml.*	*μM per ml.*
0.0–0.5	15	8.6	None	0.87
	15	8.6	0.013	4.85
0.4–0.5	15	12.5	None	0.52
	15	12.5	0.0065	2.52
	15	12.5	0.0260	5.89



It may be pointed out that other phosphomutases are known to exist (*e.g.*, those for ribose-1-phosphate and desoxyribose-1-phosphate), which may have the same mechanism of action.

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INHIBITION OF PHOSPHORYLATION BY AZIDE IN KIDNEY HOMOGENATE

Sirs:

Azide and dinitrophenol have lately come into collateral use[1] as standard reagents in the study of the biochemistry of growth. Such experiments had been mainly carried out with intact organisms or tissues.[2] In a recent communication we reported[3, 4] that in tissue homogenates dinitrophenol dissociates the hydrogen transfer reaction from the generation of phosphate bonds and makes the system apparently independent of the presence of inorganic phosphate.

TABLE I

All Warburg cups contained 1.0 ml. of washed kidney homogenate plus additions as described in our previous communication.[3] Sodium azide (Eastman, practical) was added from a side arm together with 0.5 ml. of 0.1 M $K_3Fe(CN)_6$ after flushing of the vessels with N_2. Time 5 minutes; temperature 37°.

Additions	Ferrocyanide produced*	Phosphate uptake	$P/2Fe(CN)_6^{\equiv}$
None....................	43.5	18.7	0.86
1.7×10^{-3} M NaN_3.......	46.5	6.1	0.26
3.3×10^{-3} " "	45.8	3.2	[illegible]
1.7×10^{-2} " "	41.8	1.3	0.06

All figures in micromoles.

* Ferrocyanide was measured colorimetrically with ferric iron-gum ghatti solution after Folin and Malmros (*J. Biol. Chem.*, **83**, 115 (1929)).

TABLE II

All the cups were as described above, with the exception that adenosine-5-phosphate was added only as described below. Temperature 37°; time 5 minutes.

Adenosine-5-phosphate, M................. NaN_3, M.................	0 0	0 1×10^{-3}	6.7×00^{-4} 0	6.7×10^{-4} 1×10^{-3}
Ferrocyanide produced (μM)...............	7.5	17.8	27.2	27.5

The rather striking analogy of the biological action of azide to that of dinitrophenol made it particularly desirable to attempt likewise to localize its point of attack. Preliminary results with respiring homogenates already suggested a dissociation of respiration from phosphorylation. However, the well known sensitivity of the oxygen-activating system to azide overlapped so closely with the effect on phosphorylation that this system appeared unsuited for obtaining clear cut evidence. It subsequently appeared that oxygen could be effectively replaced by ferricyanide as hydrogen acceptor, whereupon the hydrogen transfer reaction became insensitive to both cyanide and azide. Such a system appeared to be better suited for a study of the azide effect. The following experiment now shows an unambiguous interference by azide with phosphorylation, with yeast hexokinase-fructose as the phosphate bond acceptor system.

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[1] Clifton, C. E., *Advances in Enzymol.*, **6**, 269 (1946). Spiegelman, S., Kamen, M. D., and Sussman, M., *Arch. Biochem.*, **18**, 409 (1948).

[2] Hall, T. S., and Moog, F., *J. Exp. Zool.*, **109**, 339 (1949).

[3] Loomis, W. F., and Lipmann, F., *J. Biol. Chem.*, **173**, 807 (1948).

[4] Cross, R. J., Taggart, J. V., Covo, G. A., and Green, D. E., *J. Biol. Chem.*, **177**, 655 (1949).

This inhibition is easily reversible by washing the residue. A further analysis suggests that azide acts between the primarily formed phosphate bond and adenylic acid, since inorganic phosphate appears still to be essential for the hydrogen transfer reaction, while adenylic acid may at least partially be "replaced" by azide, as shown in the subsequent experiment.

Experiments in progress show that dyes like methylene blue and cresyl blue, although accelerating respiration, disrupt the link to phosphorylation and replace inorganic phosphate.[5]

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[5] The authors wish to express their thanks to Miss Helen Vavoudes for technical assistance.

THE PHEOPORPHYRIN NATURE OF CHLOROPHYLL *c*

Sirs:

Strain and coworkers[1] have definitely established the fact that there exists in the diatoms, dinoflagellates, and brown algae, besides chlorophyll *a*, another green pigment to which they have given the name *chlorophyll c*. We have recently isolated a small quantity of this pigment from a *Laminaria* species obtained from Woods Hole, and have confirmed the absorption spectrum of Strain *et al.*, which so far has been the only property identifying this pigment.

We have now found that this compound is a Mg complex, the Mg having been identified by a micro modification of the titan yellow method.[2] In contrast to the traces of acid which are sufficient to split Mg from chlorophyll *a* and *b*, the removal of Mg from this compound requires a surprisingly high acidity, in the neighborhood of 3 to 4 N HCl.

The HCl number of this compound devoid of Mg is about 12, indicating that it does not possess a phytol group. Its spectrum resembles that of a pheoporphyrin rather than a pheophorbide (*i.e.* pyrrole Ring IV is not reduced). The presence of a cyclopentanone ring is suggested by the positive phase test and the formation of a chloroporphyrin type of spectrum on treatment with methyl alcoholic HCl.

These properties of chlorophyll *c* suggest that this compound may be a modified Mg pheoporphyrin, containing an as yet unidentified chromophore group and lacking phytol. According to this interpretation the compound would then be more closely related to protochlorophyll than to chlorophyll. Further work is contemplated when more material becomes available.

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[1] Strain, H. H., and Manning, W. M., *J. Biol. Chem.*, **144**, 625 (1942). Strain, H. H., Manning, W. M., and Hardin, G., *J. Biol. Chem.*, **148**, 655 (1943).

[2] Ludwig, E. E., and Johnson, C. R., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 895 (1942).

PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

XII. PREPARATION OF 17-HYDROXYPROGESTERONE AND OTHER 17α-HYDROXY-20-KETOSTEROIDS*

Sirs:

A 17-hydroxyl group in the proper spatial configuration greatly enhances the biological activity of adrenocortical hormones compared with other steroids lacking this functional group. The partial synthesis of this important class of substances has been difficult because of the unavailability of the initial reactants. Introduction of the 17-OH group in the natural configuration has been hitherto achieved through OsO_4 oxidation of the $\Delta^{17,20}$-unsaturated steroids[1] and more recently by reduction of 16,17-epoxy-20-ketosteroids with $LiAlH_4$.[2] We wish to report a simple procedure for the direct introduction of a 17α tertiary alcohol group into the readily available 20-ketosteroids.

Treatment of the enol acetate of a 20-ketosteroid with a concentrated solution of perbenzoic acid in either benzene or chloroform results in formation of a 17-hydroxysteroid where the tertiary alcohol group occupies the desired α configuration. Oxidation of such enol acetates with CrO_3 in a biphasic system likewise effects hydroxylation at C-17 in the α configuration, although the yield is less satisfactory than that obtained with perbenzoic acid.

In a typical experiment 4.96 mM of perbenzoic acid in 8.0 ml. of chloroform were added to 3.75 mM (1.51 gm.) of amorphous 3α,20-diacetoxy-$\Delta^{17,20}$-pregnene.[3] The solution warmed spontaneously and was stored for 2 hours; titration of an aliquot indicated that approximately 90 per cent of the enol acetate had reacted. The neutral reaction product was saponified at room temperature for 30 minutes and yielded a crystalline compound melting at 193–200°. Recrystallization from acetone gave pure 3α,17α-dihydroxypregnan-20-one, m.p. 208–209.5°; $[\alpha]_D^{25} = +63°$ (ethanol). The infra-red spectrum was identical with that of an authentic

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* The work herein reported was supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, the Lillia Babbit Hyde Foundation, and the National Cancer Institute, United States Public Health Service.

[1] Serini, A., Logemann, W., and Hildebrand, W., *Ber. chem. Ges.*, **72**, 391 (1939). Sarett, L. H., *J. Biol. Chem.*, **162**, 601 (1946).

[2] Plattner, P. A., Heusser, H., and Feurer, M., *Helv. chim. acta*, **31**, 2210 (1948). Julian, P. L., Meyer, E. W., and Ryden, I., *J. Am. Chem. Soc.*, **71**, 756 (1949).

[3] Marshall, C. W., Kritchevsky, T. H., Lieberman, S., and Gallagher, T. F., *J. Am. Chem. Soc.*, **70**, 1837 (1948).

sample. This compound has been identified as a urinary metabolite by Lieberman and Dobriner.[4] The monoacetate, prepared with acetic anhydride and pyridine, melted at 198.5–199.5° and exhibited an infra-red spectrum identical with that of the known compound. Oxidation of the acetate with chromic acid yielded 3α-acetoxyetiocholan-17-one, m.p. 94–95°.

In similar fashion 3β,17α-dihydroxyallopregnan-20-one (Reichstein's compound L) was prepared from the enol acetate of allopregnanolone. The product melted at 257–259° (capillary); $[\alpha]_D^{25} = +31.7°$ (ethanol). The monoacetate melted at 188–190°; $[\alpha]_D^{23} = +16°$ (acetone).

Oxidation of 3α,17α-dihydroxypregnan-20-one with *N*-bromoacetamide yielded 17α-hydroxypregnan-3,20-dione, m.p. 215–217°; $[\alpha]_D^{25} = +53.9°$ (ethanol); $C_{21}H_{32}O_3$, calculated, C 75.82, H 9.70; found, C 76.05, H 9.60. Bromination of the diketone followed by dehydrobromination with pyridine yielded 17α-hydroxyprogesterone, m.p. 219–220°; $\epsilon_{2420} = 16{,}500$ (ethanol). The product showed no depression of melting point upon admixture with an authentic sample obtained from adrenal glands (kindly furnished by Dr. M. Kuizenga).

We have also prepared these adrenal cortical hormones with deuterium in known stable positions, *e.g.* at 11 and 12 and at 5 and 6. These compounds will be employed in metabolic studies with human subjects.

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[4] Lieberman, S., and Dobriner, K., *J. Biol. Chem.*, **161**, 269 (1945).

THE NET UTILIZATION OF AMMONIUM NITROGEN BY THE GROWING RAT*

Sirs:

While it is well known that ruminants (with the aid of rumen microorganisms) can utilize ammonium salts and urea in lieu of dietary protein,[1, 2] it has long been held that monogastric animals cannot.[1] In view of the findings of Foster, Schoenheimer, and Rittenberg[3] that dietary ammonium N^{15} is rapidly incorporated into rat tissue proteins, it seemed possible that the nitrogen of ammonium salts might be utilized to replace that of the "non-essential"[4] amino acids. This possibility has been tested with rats fed low levels of the essential[4] amino acids.

	Lot I	Lot II	Lot III	Lot IV	Lot V
N in ration, %	1.26	1.28	1.01	0.82	1.09
Weight gain during 2nd–4th wks., *gm.*	48, 43	66, 51	24, 27	29, 27	47, 48
Average daily N intake, *mg.**	126.3	153.8	106.7	67.8	137.0
" " fecal N, *mg.**	21.1	18.7	18.7	13.4	19.1
" " urinary N, *mg.**	30.6	27.6	29.3	10.2	12.7
" " " NH_4-N, *mg.**	1.6	4.0	0.6	0.3	2.2
Dietary N retained, %	59	70	65	66	77

* The nitrogen balance data were obtained for 6 consecutive days during the 4th week of the experiment.

A basal diet of amino acids, sucrose 15, corn oil 5, Phillips and Hart salt mixture 4, crystalline vitamins 0.3,[5] liver extract 1:20 (Wilson) 0.1, and dextrin to make 100 per cent was used. Vitamins A and D were given by dropper weekly.

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[1] Mitchell, H. H., and Hamilton, T. S., The biochemistry of the amino acids, New York, 571 ff. (1929).

[2] Hart, E. B., Bohstedt, G., Deobald, H., and Wegner, M. I., *J. Dairy Sc.*, **22**, 785 (1939).

[3] Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).

[4] Rose, W. C., Oesterling, M. J., and Womack, M., *J. Biol. Chem.*, **176**, 753 (1948).

[5] Betheil, J., Wiebelhaus, V. D., and Lardy, H. A., *J. Nutr.*, **34**, 431 (1947).

Lot I was fed a mixture of eighteen amino acids similar to Mixture XXIII of Rose *et al.*[4] except that the level of phenylalanine was increased 33 per cent and tyrosine was omitted. The mixture was fed at a level of 10.3 per cent of the diet. Lot II received only the essential amino acids at a level of 8 per cent and diammonium citrate (2.15 per cent) to raise the level of total nitrogen to that of Lot I. Lot III received only the essential amino acids, and Lot IV same as Lot III except that L-valine, L-isoleucine, L-threonine, and L-phenylalanine replaced the DL forms of these amino acids. Lot V received the same as Lot IV plus diammonium citrate at 2.15 per cent. The nitrogen content of the rations is shown in the table. The difference in nitrogen content between Lots III and IV and Lots II and V results from the D-amino acids present in the first of each pair. The rations were fed *ad libitum.* Two weanling male rats were used per lot. The L-amino acids not available commercially in pure form were prepared by a resolution procedure developed by Dr. D. G. Daugherty and Mr. E. A. Popenoe.

As shown in the table, diammonium citrate added to diets containing only 6.4 per cent of a mixture of essential physiologically active amino acids gave as great a growth response (Lots II and V) as did the non-essential amino acids (Lot I). That the inorganic nitrogen is being incorporated into organic forms is demonstrated by the fact that the amount of urinary ammonium nitrogen in Lots II and V is about 10 per cent of that ingested and is not far greater than the amount excreted by the rats in Lot I. Nitrogen balance studies, urinary analyses for ammonium nitrogen, and growth data indicate *a net utilization of ammonium nitrogen by the growing rat.*

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GROWTH STUDIES ON TETRAHYMENA GELEII H*

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Tetrahymena geleii H, a holotrichous protozoan, was isolated as a bacteria-free culture in 1931 by Dr. Alford Hetherington at Pacific Grove, California. The culture of the ciliate employed in the present studies was obtained in 1945 through the courtesy of Dr. George W. Kidder, who had received a culture from Dr. R. H. Hall in 1938 (2). Hetherington named this microorganism *Colpidium campylum*, but in 1940 Furgason (3) renamed it *T. geleii*, in honor of the eminent protozoologist, J. von Gelei. It was observed by Furgason that *T. geleii* and other similar organisms differed morphologically from *Colpidium* and related genera.

Investigations on the nutrition and metabolism of *T. geleii* H were initiated by Hall (4–9) in 1935 and by Kidder (10) in 1939. During the past decade, Kidder and collaborators have studied the requirements of *T. geleii* H and other strains for amino acids (2, 11–15), vitamins (16–22), carbohydrates (2, 12), purines (23), pyrimidines (23), nucleosides (23), nucleotides (23), growth factors (10, 24–28), and other substances (11, 29).

The present authors' interest in *Tetrahymena* was stimulated by the report of Kidder and Dewey (2, 18, 24) in 1944 that certain amino acids were essential for the growth of these protozoa and that bound, as well as free, amino acids were utilized. It seemed appropriate, therefore, to study further the nutrition of *Tetrahymena* and to explore the potentialities of this organism for the determination of amino acids in unhydrolyzed proteins. Although microbiological assay, isotope dilution, amino acid decarboxylase, and colorimetric and other procedures were available for the determination of free amino acids, there was no general method for the determination of bound amino acids. In 1944, Regnery (30) determined leucine microbiologically in unhydrolyzed casein with the aid of

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* Paper 51. For Paper 50, see Dunn (1). From the thesis presented by L. B. Rockland to the Graduate School of the University of California, Los Angeles, in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1948. This work was aided by grants from the National Institutes of Health, United States Public Health Service, and the University of California. The authors are indebted to Dr. George W. Kidder for cultures of *Tetrahymena* and to Jose Lieberman for technical assistance.

† Junior Research Fellow of the National Institutes of Health, United States Public Health Service, 1947–48.

leucineless Neurospora crassa. Chemical methods for the determination of certain amino acids in unhydrolyzed proteins have been reviewed by Mitchell and Hamilton (31), Block and Bolling (32), and Martin and Synge (33).

EXPERIMENTAL

Stock Cultures—Stock cultures of *T. geleii* H were maintained at room temperature (about 25°) in 18 mm. × 150 mm., cotton-plugged Pyrex test-tubes, each containing 15 ml. of broth. The composition of the broth, 2 per cent proteose-peptone (Difco) and 1 per cent glucose, was that recommended by Dr. Kidder in a private communication. The stock cultures remained viable for periods up to 100 days.

Inoculum—100 ml. of sterile stock-culture broth were sterilized in a 200 ml. Pyrex centrifuge bottle. The broth was inoculated with a single loopful of stock culture, the bottle plugged with gauze-wrapped cotton, and the inoculated broth incubated for 7 days at 25°. A rubber band was placed around the edges of the cotton plug and the bottle was centrifuged about 4 minutes at approximately 500 R.P.M. The bottle was removed from the centrifuge immediately after the rotor came to rest.[1] The plug was removed, the mouth of the bottle was flamed, about 90 per cent of the supernatant solution was decanted, approximately 90 ml. of sterile distilled water were added, and the bottle was flamed and plugged. The bottle was centrifuged and the supernatant solution decanted in the manner described. This centrifuging and washing procedure was repeated twice. The washed inoculum (about 100 ml. final volume) was allowed to stand for about 1 hour, after which the supernatant liquid containing the viable, more active protozoa was decanted into a sterile flask. The activity of the inoculum cells was checked by microscopic examination, since slow moving cells with large vacuoles probably are dying and are unsatisfactory inocula.

Inoculation Technique—The syringe assembly was removed from a Brewer automatic pipetting machine,[2] a No. 18 hypodermic needle was attached to the valve outlet, and the inlet and outlet tubes were placed in a large Pyrex test-tube capped with a heavy cotton plug. This syringe assembly was sterilized in an autoclave, allowed to cool to room temperature, and mounted on the pipetting machine. The syringe assembly was flushed with the inoculum, after which a 0.20 ml. aliquot of thoroughly mixed inoculum was delivered as rapidly as possible into each assay tube. The tubes become contaminated occasionally, since flaming them seemed impracticable; however, contamination is easily detected by visual observation after incubation or by inconsistent results from replicate tubes.

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[1] Since the viable Protozoa start to swim to the surface of the liquid immediately following centrifugation, the latter must be repeated if decantation is delayed much more than 10 seconds or the cells are dispersed by jarring the bottle. Centrifugation at too high speed or for too long a time and recentrifugation should be avoided, since the proportion of organisms killed may be greatly increased.

[2] Baltimore Biological Laboratories, Baltimore, Maryland.

TABLE I

*Basal Medium for T. Geleii H**

Component	Amount per 1000 ml. solution	Component	Amount per 1000 ml. solution
	gm.		*ml.*
Glucose	10.0	Phosphate buffer‡	7.5
Amino acid mixture (see Table II)	7.0	Folic acid solution§	1.0
	ml.	Liver extract‖	1.0
Mineral solution (see Table III)	7.5		
Vitamin Solution I (see Table IV)	6.0	Cerophyl extract¶	100.0
			mg.
Vitamin Solution II (see Table V)	7.0	Guanine chloride	50.0
		NaCl**	180
Nucleic acid hydrolysate†	1.5		

* Final solution brought to pH 7.0 with NaOH.

† The hydrolysate is a mixture containing 10.0 gm. of yeast nucleic acid (Schwarz Laboratories, New York) and 100 ml. of 0.75 N HCl autoclaved for 1 hour at 120°, 10.0 gm. of the same yeast nucleic acid and 100 ml. of 0.75 N NH_4OH autoclaved for 1 hour at 120°. These solutions were combined, brought to pH 7.0 with 0.1 N NaOH and stored in a glass-stoppered bottle in the refrigerator.

‡ Aqueous solution containing 9.6 gm. of $Na_2HPO_4 \cdot 12H_2O$ and 1.9 gm. of $NaH_2PO_4 \cdot H_2O$ per 100 ml.

§ Ethanol (50 per cent) solution containing 10 mg. of crystalline folic acid (Lederle, No. 7-5582) per 100 ml.

‖ A mixture containing 200 gm. of liver concentrate powder (Wilson, 1:20) and 4 liters of distilled water was adjusted to pH 3.0 with H_2SO_4 and filtered through Celite. 40 gm. of norit A were added, the mixture stirred for 1 hour, and the suspension filtered. The filtrate was discarded and the residual norit A eluted with 500 ml. of a 10 per cent solution of NH_4OH in 50 per cent ethanol. The ammonia was removed and the solution evaporated by passing a stream of air over the surface of the solution. The brown-colored residual solution was diluted to 250 ml. with 95 per cent ethanol and stored in a glass-stoppered bottle in the refrigerator.

¶ Prepared as described by Kidder and Dewey (2).

** Introduced through neutralization of HCl by addition of NaOH to bring the basal medium solution to pH 7.0.

Basal Medium—The composition of the basal medium employed in the present studies is shown in Tables I to V. The types and proportions of nutrients selected were determined from the results of preliminary experiments (not recorded here) on growth and acid[3] production of *T. geleii* H. The availability of a high quality basal medium made it possible to measure the response of the organism in terms of optical density of the suspensions and acid formed by the cells rather than by the laborious cell-counting methods employed previously. It is of interest that the authors' medium contained eight amino acids, two vitamins, ten mineral salts, and some other nutrient materials in addition to those present in any of the six media employed by Kidder *et al.* (2, 13–16, 19–21) since 1945. Furthermore, the majority of the constituents were present in considerably higher concentration in the authors' medium than in Kidder's media.

TABLE II

*Amino Acid Mixture**

L-Amino acid (anhydrous free base)	Amount	L-Amino acid (anhydrous free base)	Amount
	per cent		*per cent*
Alanine	1.85	Lysine†	8.09
Arginine†	4.82	Methionine‖	2.57
Aspartic acid‡	9.13	Phenylalanine‖	4.57
Cysteine†	0.83	Proline	6.37
Glutamic acid	15.3	Serine‖	5.09
Glycine	2.60	Threonine‖	5.77
Histidine§	2.68	Tryptophan	0.71
Hydroxyproline	1.81	Tyrosine	2.50
Isoleucine‖	8.17	Valine‖	8.10
Leucine	9.07		
		Total	100.0

* Amino acids (except test amino acid), ground in a ball mill, as described by Rockland and Dunn (34).

† Equivalent quantity of monohydrochloride used.

‡ Natural asparagine may be substituted for L-aspartic acid.

§ Equivalent quantity of monohydrochloride monohydrate used.

‖ DL form used in twice the specified quantity.

Microbiological Techniques—The techniques were similar to those employed previously with lactic acid bacteria. Water, standard or sample solution, and basal medium were added to 18 × 150 mm. test-tubes or 25 ml. conical flasks with the aid of a Brewer automatic pipette. The final volume in each tube or flask was 10 ml. The tubes or flasks were plugged with gauze-wrapped cotton[4] and sterilized by heating them for

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[3] Thomas (35) has shown that under anaerobic conditions *T. geleii* H produces acetic, lactic, and succinic acids.

[4] Covering each row of tubes with a tightly fitting strip of toweling has also been found satisfactory, although it is more difficult to prevent contamination by this

10 to 15 minutes at 120° in an autoclave. The tubes or flasks were inoculated as described under "Inoculation technique" and incubated at room temperature (about 25°) in a cupboard. Since there was no precise temperature control, the position of the racks was shifted twice daily in

TABLE III

Mineral Solution

Substance	Amount per 100 ml. water solution	Substance	Amount per 100 ml. water solution
Calcium acetate·H_2O	2.17 *gm.*	$ZnCl_2$	1.30 *mg.*
K_2HPO_4	0.85 "	H_3BO_3	1.20 "
$MgSO_4 \cdot 7H_2O$	0.57 "	$CuSO_4 \cdot 5H_2O$	0.57 "
KH_2PO_4	0.43 "	$CoCl_2 \cdot 6H_2O$	40.0 γ
NH_4Cl	49.0 *mg.*	KI	26.5 "
Ferric citrate	27.5 "	NaF	8.5 "
$MnCl_2 \cdot 2H_2O$	11.0 "	HCl (11.7 N)	3.5 *ml.*

TABLE IV

*Vitamin Solution I**

Vitamin	Amount per 100 ml. 50 per cent ethanol solution
	mg.
Biotin	2.00
Pyridoxine·HCl	2.00
p-Aminobenzoic acid	5.00
Thiamine chloride	10.0
Riboflavin	40.0
Nicotinic acid	50.0
Pantothenic acid†	70.0

* Stored in an amber, glass-stoppered bottle in the refrigerator.

† An equivalent quantity of calcium or sodium salt may be used.

TABLE V

*Vitamin Solution II**

Vitamin	Amount per 100 ml. 40 per cent ethanol solution
	gm.
Choline chloride	1.00
i-Inositol	1.00
Ascorbic acid	0.100

* Stored in a glass-stoppered bottle in the refrigerator.

stant, temperature. The optical density and acid of the cell suspensions were measured by the authors' previously described turbidimetric and acidimetric procedures (36, 37).

TABLE VI

Titration and Turbidity Data Showing Response of T. Geleii H to Four Amino Acids in Tubes and Flasks at Different Incubation Times

Amino acid	Container	Incubation time	Titration		Turbidity	
			Half maximum response*	Sensitivity index†	Half maximum response‡	Sensitivity index§
		days	*ml.*			
L-Histidine	Upright tubes	12	4.30	5.5	‖	
		17	4.30	5.3	‖	
		24	4.85	5.9	‖	
	Slanted tubes	12	4.92	4.1	1.79	25
		17	4.77	5.1	1.88	26
		24	3.80	6.4	2.16	41
	Flasks	12	‖		2.10	35
		17	‖			
		24	‖		2.25	40
L-Tryptophan	Upright tubes	10	3.35	14.5	‖	29
		14	3.95	20	‖	61
		19	4.25	21	‖	61
	Slanted tubes	10	4.00	6.7	1.31	49
		14	4.65	11.1	1.70	57
		19	4.38	13.3	1.84	55
	Flasks	10	‖		1.81	52
		14	‖			
		19	‖		2.00	49
L-Isoleucine¶	Upright tubes	9	3.25	1.7	0.67	5.8
		13	3.75	2.3	0.97	10.5
		20	4.97	3.7	0.86	6.5
L-Lysine	Upright tubes	9	‖		0.63	2.2
		13	‖		0.85	5.4
		20	‖		1.09	8.9

* Ml. of 0.01 N NaOH.

† Ml. of 0.01 N NaOH at half maximum response per microgram of amino acid $\times 10^3$.

‡ Optical density at half maximum × 10.

§ Optical density at half maximum per microgram of amino acid $\times 10^4$.

‖ Standard curves irregular.

¶ Twice the quantity of DL-isoleucine used.

EXPERIMENTAL RESULTS

The response of *T. geleii* H to tryptophan and histidine in upright and slanted tubes and in flasks at incubation times from 10 to 24 days in terms of turbidity (optical density) and acidity is shown in Table VI. Similar

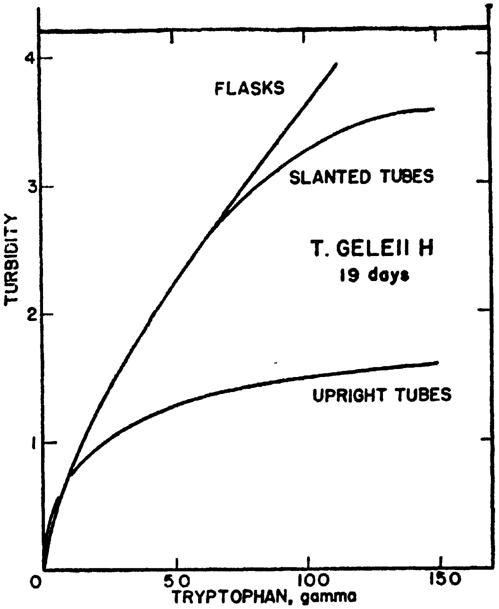
FLASKS
SLANTED TUBES
UPRIGHT TUBES
T. GELEII H
19 days
TURBIDITY
4
3
2
1
0
50
100
150
TRYPTOPHAN, gamma

FIG. 1

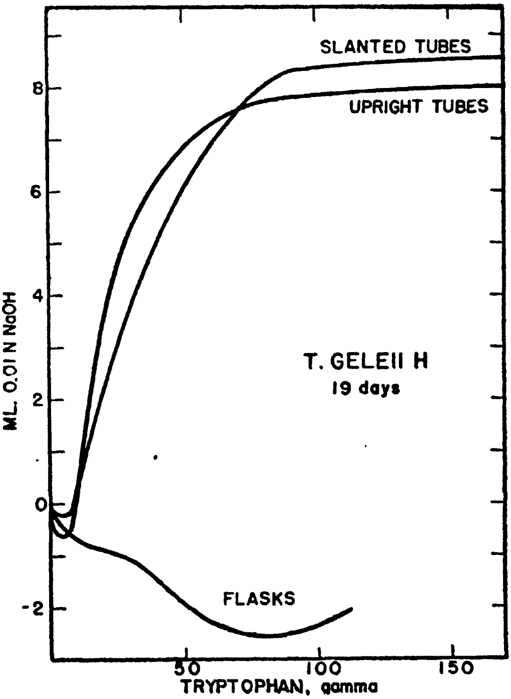
SLANTED TUBES
UPRIGHT TUBES
FLASKS
T. GELEII H
19 days
ML. 0.01 N NaOH
8
6
4
2
0
-2
50
100
150
TRYPTOPHAN, gamma

FIG. 2

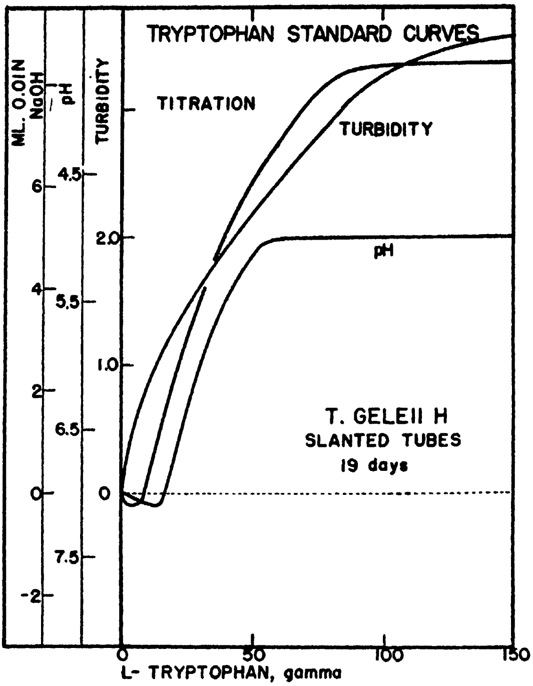
TRYPTOPHAN STANDARD CURVES
ML. 0.01N NaOH
pH
TURBIDITY
TITRATION
TURBIDITY
pH
T. GELEII H
SLANTED TUBES
19 days
6
4
2
0
-2
4.5
5.5
6.5
7.5
2.0
1.0
0
0
50
100
150
L- TRYPTOPHAN, gamma

FIG. 3

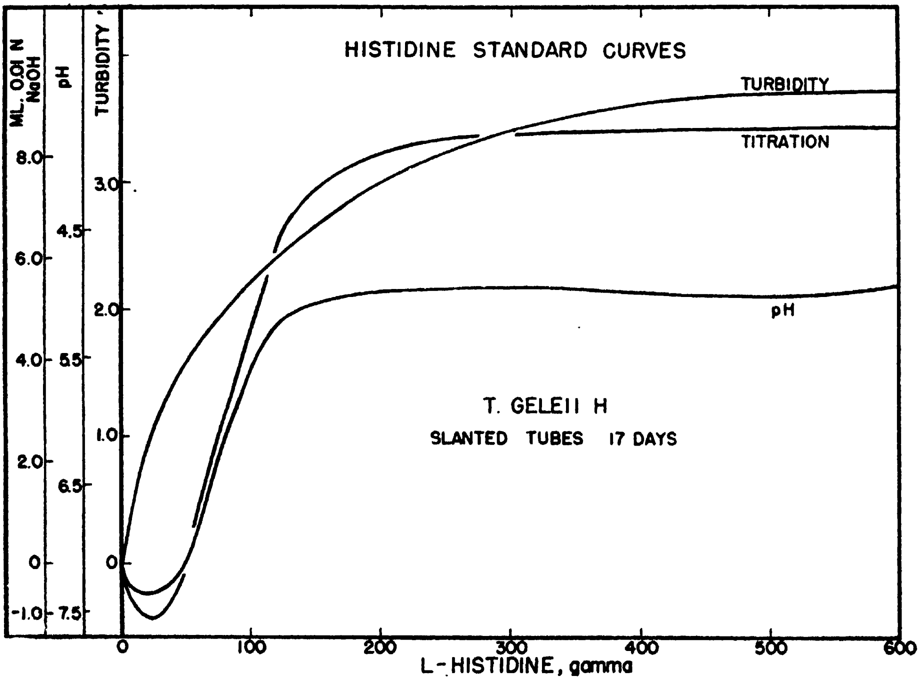
HISTIDINE STANDARD CURVES
ML. 0.01 N NaOH
pH
TURBIDITY
TURBIDITY
TITRATION
pH
T. GELEII H
SLANTED TUBES 17 DAYS
8.0
6.0
4.0
2.0
0
-1.0
4.5
5.5
6.5
7.5
3.0
2.0
1.0
0
0
100
200
300
400
500
600
L-HISTIDINE, gamma

FIG. 4

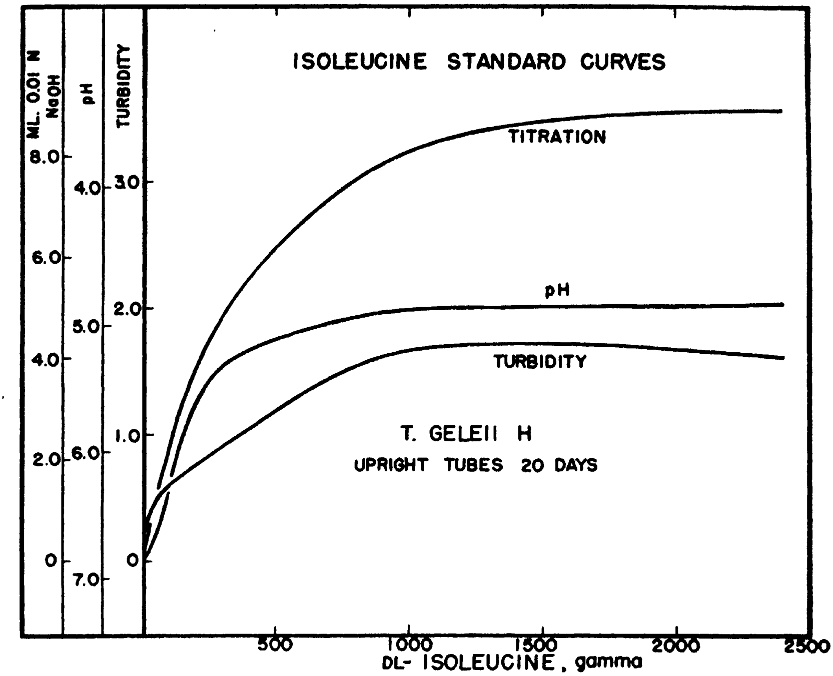
ISOLEUCINE STANDARD CURVES
ML. 0.01 N NaOH
pH
TURBIDITY
8.0
6.0
4.0
2.0
0
4.0
5.0
6.0
7.0
3.0
2.0
1.0
0
TITRATION
pH
TURBIDITY
T. GELEII H
UPRIGHT TUBES 20 DAYS
500
1000
1500
2000
2500
DL- ISOLEUCINE, gamma

FIG. 5

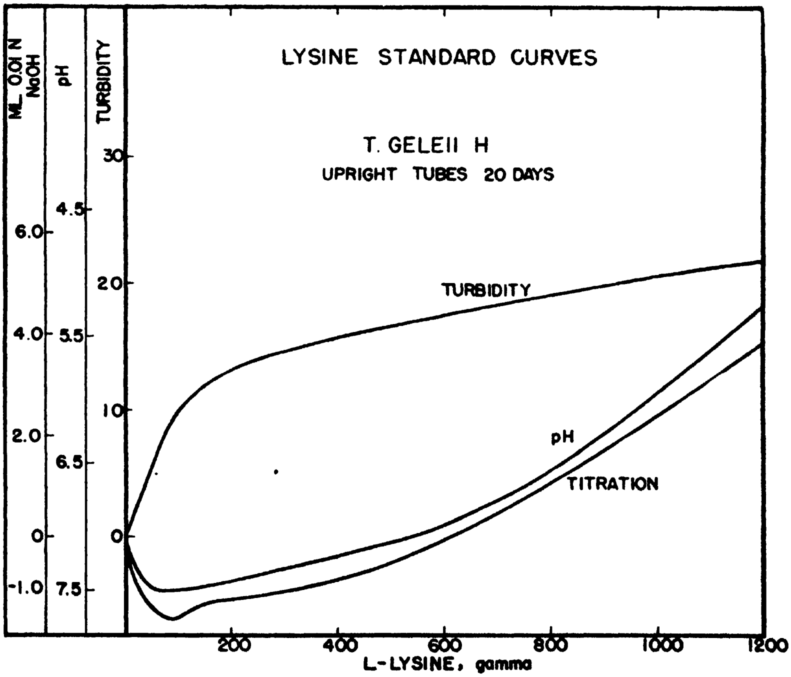
LYSINE STANDARD CURVES
ML 0.01 N NaOH
pH
TURBIDITY
T. GELEII H
UPRIGHT TUBES 20 DAYS
6.0
4.0
2.0
0
-1.0
4.5
5.5
6.5
7.5
30
20
10
0
TURBIDITY
pH
TITRATION
200
400
600
800
1000
1200
L-LYSINE, gamma

FIG. 6

studies on isoleucine and lysine in upright tubes are given in Table VI. Standard curves showing the turbidimetric response of *T. geleii* H to tryptophan in flasks, slanted tubes, and upright tubes at 19 days incubation time are shown in Fig. 1 and the acidimetric response in Fig. 2.

It is of interest that alkaline mixtures were formed when the organism was grown at low levels of tryptophan in slanted or upright tubes as well as at all levels of tryptophan in flasks. Cell proliferation was approximately the same in slanted tubes and flasks but acid production was greater in slanted and upright tubes than in flasks. The reasons for these results are not entirely clear, but it may be noted that the air-liquid interfaces were approximately equal in the slanted tubes and flasks and were greater in these containers than in the upright tubes. Presumably the oxygen tension was less in the solutions of greater than those of lesser depth, due possibly to the utilization of oxygen near the surface of the liquids by this essentially aerobic organism.

Standard curves for the four amino acids investigated are shown in Figs. 3 to 6. Experiments are in progress on the development of assay procedures for the determination of these amino acids.

SUMMARY

A basal medium containing extracts of liver and Cerophyl powders, in addition to chemically defined components, has been developed on which *Tetrahymena geleii* H grew well and produced a relatively large amount of acid. Growth and acid production by this organism have been studied in flasks, upright tubes, and slanted tubes over incubation periods up to 24 days. The inverse relation found between growth and acid production under some conditions may be related to the area of the liquid-air interfaces and to the volumes of unexposed liquids. The observation of Kidder and Dewey (2) has been confirmed that isoleucine, lysine, histidine, and tryptophan are essential for the protozoan, and standard curves have been obtained for these amino acids. Assay procedures for the determination of some of these amino acids are under investigation.

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STUDIES ON PROTEINS FROM BOVINE COLOSTRUM

III. THE HOMOLOGOUS AND HETEROLOGOUS TRANSFER OF INGESTED PROTEIN TO THE BLOOD STREAM OF THE YOUNG ANIMAL*

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It has been adequately demonstrated that the new-born calf and kid acquire antibody proteins from ingested colostrum (1). These immune proteins appear in the blood stream of the young animal in a form immunologically indistinguishable from that in the colostrum (1). This apparent passage of intact protein through the gastrointestinal tract of the calf and kid is characteristic of the first few days of life (1, 2).

The proteins of colostrum whey of the cow, goat, and pig are similar in that the principal fraction is globulin in nature (arbitrarily called "immune" globulin) with a low electric mobility (3, 4). The "immune" globulins of cow colostrum have been reported (4) to migrate in an electric field at a faster rate than the corresponding proteins of goat and pig colostrum. This difference in mobility has been used to study, by electrophoresis, the apparent gastrointestinal absorption of these colostrum proteins by the young kid. Further, immunological tests have been employed to study the possible transfer of normal milk "immune" protein from the gastrointestinal tract to the blood of the new-born calf. The results of both the electrophoretic and the immunological studies are reported here.

Methods

Colostrum samples were taken from the goat, cow, and pig immediately following parturition. Each sample was divided into two portions. One portions was fed *ad libitum* to a newly dropped kid. Electrophoretic analyses were performed in duplicate on the second portion of each colostrum whey after dialysis at 0°, first against several changes of saline, then finally against two changes of the barbiturate-citrate buffer used previously (2). Blood serum samples were taken from the jugular vein of each kid at birth and 24 hours after feeding and analyzed electrophoretically. The results were computed as in earlier studies (2).

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Three groups of new-born calves were separated from their dams before nursing and treated as follows: One group (control) was fasted 24 hours, the second group was fed milk from cows in mid-lactation, while the third was fed cow colostrum. The calves were bled from the jugular vein at birth, then again at 24 hours, and the serum was separated for the immunological studies.

Rabbits were immunized to the purified cow colostrum pseudoglobulin described previously (5). Serum from the immune rabbits (two to three per group) was collected and pooled. The possible presence of specific antigen proteins in the sera of the calf was studied by conventional precipitin tests. Various dilutions of the sera under test were placed in layers above the immune serum and turbidity was estimated visually after a 2 hour incubation period. In check determinations, turbidity was also estimated in the Beckman spectrophotometer at 660 mμ.

Results

Colostrum Feeding—The various colostrum whey electrophoretic patterns were observed to be similar to those described by Deutsch (4), and are therefore not presented in detail; however, the percentage and mobility of the "immune" component of each whey studied is given in Table I. The principal component of cow colostrum exhibited decidedly greater mobility (-2.7×10^{-5} sq. cm. per volt per second) than either of the corresponding components from goat and pig colostrum. It was not possible to distinguish between the "immune" components of goat and pig colostrum electrophoretically.

The serum of the kid at birth contains very little, if any, protein corresponding to the adult γ-globulin fraction (Fig. 1). This may perhaps be inferred from an analogy to results of the studies with the calf (6). Following ingestion of each of the three colostrum milks studied, there was a striking increase in the "immune" proteins of the goat serum. In the case of the animal receiving the cow colostrum, half (49.8 per cent) of the serum proteins was acquired from the ingested milk, while in the animals fed goat and pig colostrum, this newly acquired protein amounted to 40.2 and 26.7 per cent, respectively, of the serum proteins. As *ad libitum* feeding was permitted, it is not possible to compare the magnitude of the serum changes between the various animals, following the ingestion of colostrum from the different sources.

The electrophoretic mobility of the newly acquired protein in the kid serum in all cases was identical to the mobility of the principal whey component of the ingested colostrum (Table I). From an electrophoretic standpoint, no alteration in the protein appears to have occurred in the change from colostrum to the blood of the kid. The similarity of the colostrum "immune" proteins from the various sources studied is emphasized by the striking change in the serum of the new-born goat following their

Table I

Absorption of Colostrum Proteins by New-Born Goat As Measured by Electrophoresis

The electrophoretic analyses were performed at 1° in a barbiturate-citrate buffer at pH 8.6 and ionic strength 0.088. Mobilities are negative in sign and are expressed as sq. cm. per volt per second × 10^{-5} and were calculated with reference to the salt boundary.

Source of colostrum		Goat	Cow	Pig
Principal colostrum component	% of whey proteins	50.2	71.0	63.0
	Mobility	2.0	2.7	2.0
New component in kid serum	% of serum proteins	40.2	49.8	26.7
	Mobility	2.2	2.8	2.0

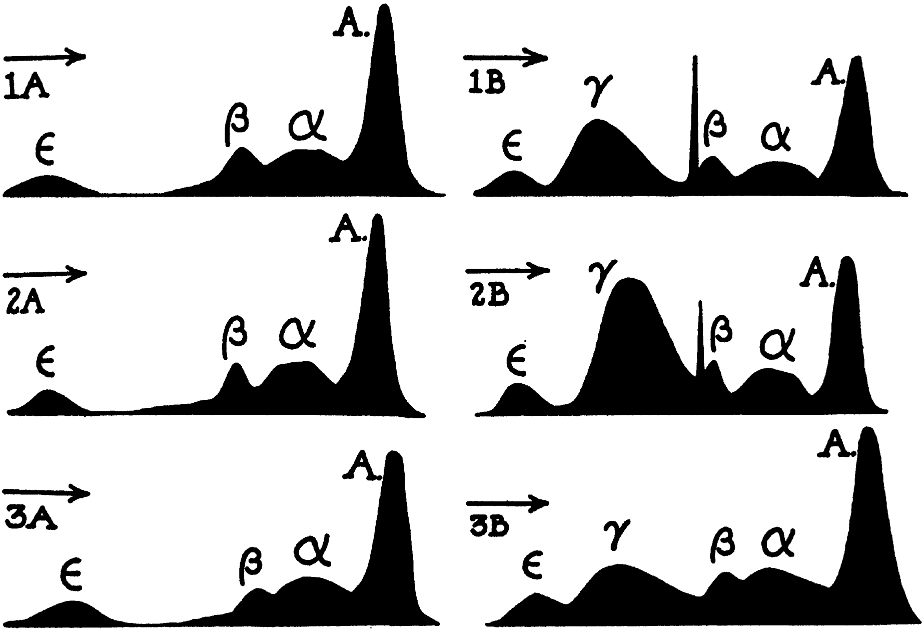

Fig. 1. Electrophoresis patterns of new-born goat serum (*A*) prior to and (*B*) 24 hours after the ingestion of goat (1), cow (2), or pig (3) colostrum. The experiments were conducted for 120 minutes in a barbiturate-citrate buffer at pH 8.6.

ingestion; this similarity is evident in spite of the mobility differences. The young animal demonstrates considerable selectivity in this reaction to ingested proteins as evidenced by the failure of other colostrum and milk proteins to appear in the blood stream as shown by the electrophoretic studies.

Immunological Studies—Even before the ingestion of milk, the serum of the new-born calf gave decided evidence for the presence of protein immunologically similar to cow colostrum pseudoglobulin (Table II). This finding is unusual inasmuch as numerous workers (1) have clearly shown that specific antibodies are not present in calf serum until after colostrum feeding. The relation of this protein to the "immune" proteins and its position in the serum electrophoretic pattern is therefore of considerable interest; however, until isolation of this protein can be accomplished, the electrophoretic serum fraction with which it is associated remains a matter of speculation.

TABLE II

Absorption of Proteins by New-Born Calf As Measured by Immunological Assay

1:10 serial dilutions of the calf sera were made. + indicates a positive turbidity, ± indicates a questionable turbidity, and − is used when no turbidity was visually evident when the diluted sera were placed in layers above the immune sera.

No. of calves	Material fed	Time	Serum dilution					
			Serum	1	2	3	4	5
3	Control	Birth	+	+	±	−	−	−
		24 hrs.	+	+	±	−	−	−
3	Normal milk	Birth	+	+	±	−	−	−
		24 hrs.	+	+	+	+	±	−
1	Colostrum	Birth	+	+	±	−	−	−
		24 hrs.	+	+	+	+	+	±

Immunological tests indicated that the serum of the young calf acquires "immune" protein from the ingestion of normal milk (Table II). The suggested (7) similarity of globulins of colostrum and normal milk is thus further indicated. The magnitude of this increase is small, however, as evidenced by the failure of electrophoretic procedures to detect any change in calf serum following the ingestion of normal milk (2).

SUMMARY

New proteins appear in the blood stream of young kids following the ingestion of goat, cow, and pig colostrum. No change in these proteins, measurable by electrophoresis, appears to have occurred during their passage from the colostrum to the blood stream of the kid.

The serum of the new-born calf contains small amounts of proteins immunologically similar to colostrum "immune" proteins.

An increase in serum "immune" proteins of the young calf resulted from the ingestion of normal milk.

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PREPARATION OF CYSTINE FROM RADIOACTIVE SULFUR*

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(Received for publication, December 16, 1948)

Small amounts of cystine labeled with S^{35} and of high specific activity were needed for our biological studies. Several methods were available for the synthesis of cystine (1–5), but each of these would require more study. Two methods have been used previously for the preparation of radioactive cystine, the methods of Wood and du Vigneaud (2, 6) and of Tarver and Melchior (3). Neither of these appeared directly applicable to the small scale production at the high level of radioactivity we required, or convenient for the recovery of unused labeled sulfur.

One of us (J. L. W.) had previously prepared benzanthrylcysteine by direct condensation of α-amino-β-chloropropionic acid with benzanthryl mercaptan in aqueous alkaline solution (7). With an improved preparation of benzyl mercaptan available (8) it has been possible to apply the analogous reaction to direct synthesis of radioactive benzylcysteine (and cystine from serine) and 16 mg. of free sulfur labeled with S^{35}. The reactions involved are illustrated. The process can be carried to completion in a test-tube connected to a gas-washing train (Fig. 1).

Benzylcysteine is the intermediate of choice, since it is the starting material for the resolution of cystine into its enantiomorphs (9, 10) and for the preparations of many derivatives. The experimental part describes the new features of the method in the necessary detail for their duplication. About half of the radioactive sulfur is incorporated into benzylcysteine. The rest remains with specific activity undiminished in a small volume of solvents for easy recovery as sulfate.

EXPERIMENTAL

Particular note should be made of the ease with which peroxides or air oxidizes benzyl mercaptan in alkaline solution. All operations with radioactive sulfur should be carried out in a well ventilated hood. We have observed under a variety of conditions, with non-radioactive materials, that *S*-benzyl derivatives such as benzylcysteine release enough volatile products to cause a taste sensation.

α-Amino-β-chloropropionic Acid—6 gm. of serine methyl ester hydro-

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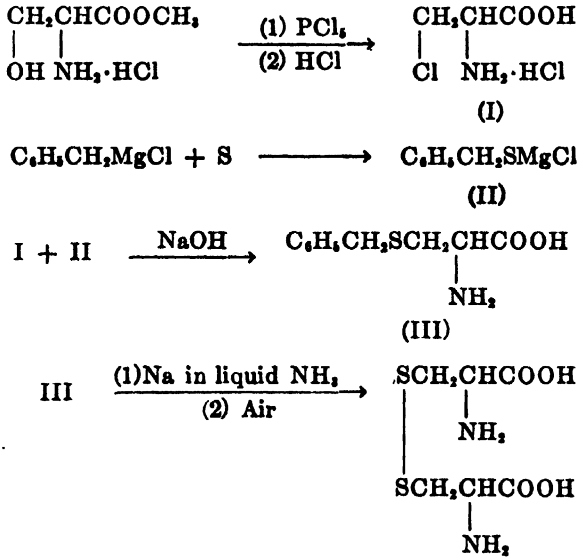
chloride[1] (11) were finely powdered and suspended in 50 ml. of dry chloroform in a 125 ml. glass-stoppered Erlenmeyer flask. Four glass beads and 9.0 gm. of powdered phosphorus pentachloride were added. The mixture was cooled in an ice bath, then allowed to warm with vigorous shaking until the evolution of hydrochloric acid indicated reaction to be occurring. The shaking was continued until all of the serine methyl ester hydrochloride had dissolved, the temperature being regulated by occasional immersion in the ice bath to keep the rate of reaction under control.

The solution was filtered into a 250 ml. suction flask. Dry diethyl ether was cautiously added to the filtrate until precipitation began. Dry petroleum ether was then added slowly until all material had separated. The product generally separated in crystalline form. If it did not, the first oil obtained was rubbed with a glass rod with cautious increments of petroleum ether until crystallization began. The product was cooled in an ice bath for 15 minutes and then filtered. Part of the crystals adhered to the flask. These were washed *in situ* with petroleum ether and this was poured over the material on the funnel until no odor of phorphorus oxychloride remained. The solid was washed out of the suction flask with 20 per cent hydrochloric acid. The material in the funnel was added and the solution (60 ml.) was heated for 1 hour at 100°.



$$\underset{\text{OH}}{\text{CH}_2}\underset{\text{NH}_3\cdot\text{HCl}}{\text{CH}}\text{COOCH}_3 \xrightarrow[\text{(2) HCl}]{\text{(1) PCl}_5} \underset{\text{Cl}}{\text{CH}_2}\underset{\text{NH}_3\cdot\text{HCl}}{\text{CH}}\text{COOH} \quad \text{(I)}$$

$$\text{C}_6\text{H}_5\text{CH}_2\text{MgCl} + \text{S} \longrightarrow \text{C}_6\text{H}_5\text{CH}_2\text{SMgCl} \quad \text{(II)}$$

$$\text{I} + \text{II} \xrightarrow{\text{NaOH}} \text{C}_6\text{H}_5\text{CH}_2\text{SCH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH} \quad \text{(III)}$$

$$\text{III} \xrightarrow[\text{(2) Air}]{\text{(1)Na in liquid NH}_3} \begin{array}{l} \text{SCH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH} \\ | \\ \text{SCH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH} \end{array}$$

The hydrochloric acid solution was evaporated to dryness *in vacuo*. About 10 ml. of benzene were added and the mixture was again evaporated to dryness. The residue was crystalline. It was dissolved in dry methanol and filtered. The methanol was evaporated to a sirup, and dry acetone was added to produce incipient cloudiness. When crystallization began, ethylene chloride was added. The product was cooled in an ice bath and then was filtered and washed with acetone. The weight of α-amino-β-chloropropionic acid hydrochloride was 2.3 gm. When immersed in the bath at 165° it melted at 172–174° (corrected) with decomposition.

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[1] We are indebted to Merck and Company, Inc., for a generous supply of serine.

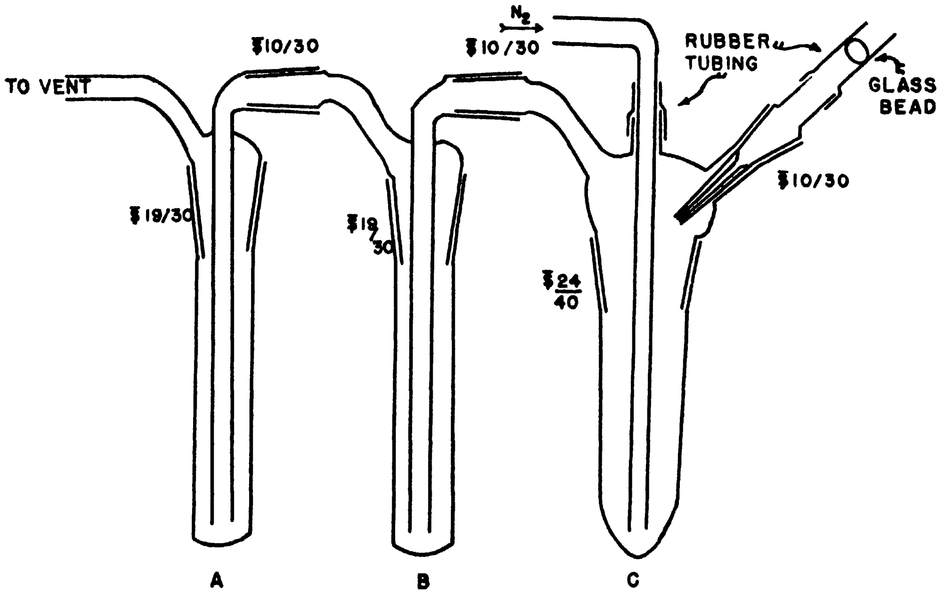

FIG. 1. Gas-washing train. *A* and *B*, safety tubes charged with 1 ml. of 5 N sodium hydroxide; *C*, reaction tube, 20 × 120 mm. The dropper on *C* contained α-amino-β-chloropropionic acid hydrocloride solution.

This product was sufficiently pure for the synthetic purposes. It was stored in a refrigerator over calcium chloride. After some months a sample contained methanol-insoluble material. It was satisfactorily purified by crystallization from a methanol-acetone-ethylene chloride mixture.

Benzylcysteine from Radioactive Sulfur—A solution of 16 mg. of sulfur in xylene was prepared by oxidizing radioactive sulfide ion[2] with iodine in potassium iodide as described previously (8). The xylene was concentrated by boiling to about 2 ml. in a 24/40 standard taper test-tube (Fig. 1). Benzyl magnesium chloride (5 ml. of 0.5 M solution in benzene (12)) was added to the cold solution; it was stoppered and allowed to stand overnight. The suspension was stirred up with 5 ml. of peroxide-free petroleum ether and then was centrifuged. The supernatant was decanted and the residue was again washed with peroxide-free petroleum ether, which was decanted. The tube containing the residue was placed on the gas-washing train illustrated in Fig. 1 and a stream of nitrogen was passed through. The tube was cooled in an ice bath and 1 ml. of 5 N sodium hydroxide was added. Stirring was effected by a stream of nitrogen from the inlet tube which extended into the test-tube. The temperature was then raised to 50° and 240 mg. of α-amino-β-chloropropionic acid hydrochloride in 7 drops of water were added over a period of 5 minutes. The suspension was maintained at 50° for 45 minutes with nitrogen stirring, and then an excess of glacial acetic acid was added. The product was overlaid with petroleum ether to retain unchanged benzyl mercaptan and then was collected on a sintered glass funnel. The crystals on the funnel were washed with a few drops of ice water, with 95 per cent ethanol, and finally with ether. The dried product weighed 40 to 46 mg., which was 38 to 44 per cent of the theoretical amount. It was sufficiently pure for dilution or for further synthesis without recrystallization. The melting point was 213–214° with decomposition.

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[2] Radioactive sulfur was obtained from the Oak Ridge National Laboratory under allocation from the United States Atomic Energy Commission, in separated form as H_2SO_4.

$C_{10}H_{13}O_2NS$.	Calculated.	N 6.63, S 15.2
(211.1)	Found.	" 6.66, " 14.7
		" 6.56, " 14.9

Typical preparations have yielded benzyl-DL-cysteine having radioactivity of 20, 50, and 250 counts per second per microgram of sulfur when measured with a thin window (2.1 mg. per sq. cm.) Geiger-Müller tube.

All residual solutions were combined with sodium hydroxide in excess. A few drops of 30 per cent hydrogen peroxide were added, and the solutions were evaporated preparatory to fusion of the residue with sodium peroxide and recovery of the sulfur as barium sulfate according to the method of Bailey (13).

As a further check on the identity of the product, 21 mg. of the synthetic *S*-benzyl-DL-cysteine were acetylated with acetic anhydride in alkaline solution. 24 mg. of the acetyl derivative, m.p. 156.5–157.5°, were obtained and shown to be identical with an authentic sample of *N*-acetyl-*S*-benzyl-DL-cysteine (14).

Radioactive Cystine[3]—A sample of radioactive *S*-benzyl-DL-cysteine was converted to *S*-benzyl-L-cysteine by isotopic dilution as described in the accompanying paper (9). 248 mg. of *S*-benzyl-L-cysteine (0.24 count per second per microgram of sulfur) were treated with sodium in dry liquid ammonia according to the method of Wood and du Vigneaud (10). The product was oxidized with air to yield 109 mg. of radioactive L-cystine (0.27 count per second per microgram of sulfur) or 77 per cent of the theoretical amount.

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[3] This preparation was carried out by Dr. H. R. Gutmann.

SUMMARY

A method for the preparation of benzyl-DL-cysteine from 16 mg. of radioactive sulfur has been presented. This product may be converted to optically inactive cystine, or resolved into benzyl-L- and benzyl-D-cysteine.

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RADIOACTIVE L-CYSTINE AND D-METHIONINE. A STUDY OF THE RESOLUTION OF RADIOACTIVE RACEMATES BY ISOTOPIC DILUTION*

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(Received for publication, December 16, 1948)

The methods of synthesis necessary to produce a radioactive asymmetric organic compound from an inorganic irradiation product generally lead to a racemic mixture. The course of many biological reactions is characterized by a stereochemical specificity, however, which makes it often desirable to use optical isomers of the labeled compound instead of the racemic mixture for experimental work. The resolution of the racemate for this purpose must be complete. If the resolution has not been complete, and the isomer used retains contaminating radioactivity in the form of its enantiomorph, the radioactivity found in various intermediates and products may represent more than one metabolic pathway.

For studies involving the sulfur-containing amino acids, radioactive, optically pure isomers of cystine and methionine were required. Methods were available for the resolution of benzyl-DL-cysteine (1) and benzyl-DL-homocysteine (2) which are used in the preparation of the optical isomers of cystine and methionine respectively. However, it was recognized that these methods are controlled by the measurement of optical activity which is too gross to detect the presence of a minute amount of labeled D isomer in an L fraction. Furthermore, the more sensitive radioactivity measurements could not be used to demonstrate the removal of D isomer from the L by fractional crystallization, since both isomers would have the same specific radioactivity.

The preparation of labeled optical isomers of cystine and methionine by isotopic dilution offered a solution to this complication. When a radioactive racemate is diluted by the addition of a large amount of one optical isomer, a difference in the specific radioactivities of the enantiomorphs is produced. Fractional crystallization to isolate a single, labeled enantiomorph can then be followed by radioactivity measurements. For example, a radioactive racemate is dissolved and the solution is then supersaturated with respect to the L form by the addition of a large amount

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* This work was supported by grants from the American Cancer Society and the Rockefeller Foundation.

of unlabeled L isomer. A solid phase can then be crystallized in the form of pure L isomer which is labeled with the major portion of the radioactive L molecules from the racemate. If the L preparation is contaminated by a trace of radioactive D molecules, the specific radioactivity of the material will decrease with successive recrystallizations as the L isomer is purified. The specific radioactivity will reach a constant value when all the radioactive D molecules have been removed. The experimental procedure is illustrated by the following examples which lead to radioactive L-cystine and radioactive D-methionine.

Solubility determinations, carried out with benzyl-L-cysteine and benzyl-DL-cysteine, showed the concentration in a saturated aqueous solution of pH 6 to 7 at ice bath temperature to be approximately 6 and 2 mg. per ml., respectively. Labeled benzyl-DL-cysteine was prepared from radioactive sulfur (3) with a specific activity which was 100 times greater than required for experimental purposes. The product was diluted 66-fold with non-radioactive benzyl-L-cysteine. The mixture was recrystallized from enough solution to retain the benzyl-DL-cysteine in the mother liquors. Due to the exchange of L molecules which occurred when the benzyl-DL- and benzyl-L-cysteines were dissolved together, the crystals of benzyl-L-cysteine, which were obtained, contained the major portion of the radioactive L molecules from the racemate. In order to improve the completeness of the separation, the product was recrystallized until the specific radioactivity no longer changed from fraction to fraction. To conform to the criteria of purity discussed below, the recrystallized benzyl-L-cysteine was converted either to *N*-acetyl-*S*-benzyl-L-cysteine or to L-cystine. These derivatives had the same specific radioactivity as the materials from which they were made.

Benzyl-DL-homocysteine was prepared from radioactive sulfur on a micro scale (1 mM) by adapting the procedure of Snyder and Chiddix (4). By omitting the isolation of intermediates, the procedure for the radioactive preparations is simplified with no sacrifice in yield. The radioactive DL mixture was diluted with 10 times its weight of non-radioactive benzyl-D-homocysteine. Recrystallization of the mixture from enough water to retain all of the DL form yielded pure, labeled benzyl-D-homocysteine. A sample was converted to the *N*-acetyl derivative without altering the specific radioactivity of the sulfur. Pure, labeled D-methionine was likewise available from the benzyl-D-homocysteine (2).

EXPERIMENTAL

The solubilities of racemic mixtures and isomers were determined on saturated solutions. The compounds were dissolved in dilute hydrochloric acid and precipitated by adjusting the solution to pH 6 to 7 by addition of dilute sodium hydroxide. After cooling at ice bath temperature, the mother liquors were analyzed for nitrogen by the micro-Kjeldahl procedure.

For radioactivity determinations the compounds were combusted by the micro-Carius or perchloric acid procedure and precipitated as benzidine sulfate by the methods previously described (5). All measurements were corrected for radioactive decay and for self-absorption by the sample.

Benzyl-L-Cysteine and L-Cystine—A mixture of 15 mg. of radioactive benzyl-DL-cysteine and 488 mg. of unlabeled benzyl-L-cysteine was crystallized by dissolving in hot dilute hydrochloric acid followed by neutralization with sodium bicarbonate solution (Table I). The crystals were collected, washed with ice water, ethanol, and ether, and dried over phosphorus pentoxide. Since the radioactivity was not changed by recrystallization, the product was converted to L-cystine by treatment with sodium in liquid ammonia followed by air oxidation (1). 248 mg. of benzyl-L-cysteine yielded 109 mg. of L-cystine which, after recrystallization, had an optical rotation which agrees with that reported in the literature, $[\alpha]_D^{20} = -223°$ (6). The chemical reaction did not alter the specific radioactivity of the sulfur.

TABLE I

Preparation of Radioactive L-Cystine from Radioactive Benzyl-DL-cysteine

Compound recrystallized	Fraction No.	Weight of sample used	Volume	Recovery	Specific radioactivity of sulfur	Estimated loss of D isomer
		mg.	*ml.*	*mg.*	*counts per sec. per γ*	*per cent*
Benzyl-L-cysteine	1	503*	7.5	362	0.23	100
	2	361	10.4	249	0.24	100
L-Cystine	1	248†		109		
	2	109		92	0.27	

* This sample consisted of 7.5 mg. each of labeled benzyl-L-cysteine and benzyl-D-cysteine in DL mixture diluted with 488 mg. of unlabeled benzyl-L-cysteine. The dilution factor for the L isomer was 66.1. The specific radioactivity before dilution was 20.9 counts per second per microgram of S.

† This is expressed as benzylcysteine equivalent to 141 mg. of L-cystine.

In another experiment a mixture of 34 mg. of crude radioactive benzyl-DL-cysteine and 500 mg. of inactive benzyl-L-cysteine was dissolved in 0.1 N hydrochloric acid, and 0.1 N sodium hydroxide was added until the pH was adjusted between 6 and 7. The solution was cooled overnight in an ice bath. The crystals were collected, washed with 1 ml. of cold water and with ethanol, and dried over phosphorus pentoxide *in* *vacuo* at 75° for 1 hour. The yield of material was 451 mg. Table II shows the progress of purification with recrystallization. As soon as sufficient material had been lost for complete retention of the D isomer by the mother liquors (Fraction 3) the acetyl derivative was prepared. However, radioactivity determinations disclosed that the fractionation of the radioactive cysteines had not been completed at this point. Thus the specific activity of the acetyl derivative was less than that of the third fraction. Two recrystallizations of the acetyl derivative from dilute alcohol did not change its specific radioactivity. The benzyl-L-cysteine was therefore twice more recrystallized, the specific activity was found to be the same as that of the acetyl derivative, and the resolution was considered complete. Furthermore, the product of the dilution factor, 30.4, times the final specific activity, 2.13 counts per second per microgram of sulfur, was much less than the specific activity of the original material. This indicated that the resolution had removed a small amount of a sulfur compound of high specific radioactivity which had contaminated the original DL sample.

TABLE II

*Preparation of Radioactive Benzyl-*L*-cysteine and N-Acetyl-S-benzyl-*L*-cysteine from Radioactive Benzyl-*DL*-cysteine*

Compound recrystallized	Fraction No.	Weight of sample used	Volume	Recovery	Specific radioactivity of sulfur	Estimated loss of D isomer
		mg.	*ml.*	*mg.*	*counts per sec. per γ*	*per cent*
Benzyl-L-cysteine	1	534*	11	451	3.33	43
	2	447	7	402	2.90	64
	3	400	6	346	2.60	78
	4	83	3	52		
	5	49	4	28	2.08	102
N-Acetyl-*S*-benzyl-L-cysteine	1	250		246	2.17	
	2	240		220	2.08	
	3				2.18	

* This sample consisted of 17 mg. each of labeled benzyl-L-cysteine and benzyl-D-cysteine in DL mixture diluted with 500 mg. of non-radioactive benzyl-L-cysteine, a dilution factor of 30.4 for the L isomer. The specific radioactivity before dilution was 140 counts per second per microgram of S. The theoretical weight loss required for resolution of isomers was estimated from the solubility determinations to be 136 mg.

In an earlier experiment, 17 mg. of labeled benzyl-DL-cysteine diluted with 200 mg. of non-radioactive benzyl-L-cysteine were dissolved in hydrochloric acid and crystallized by adding ammonium hydroxide. A determination of the optical rotation indicated that the compound was optically pure; however, the specific radioactivity of the sample was reduced by recrystallization of the compound in the form of the hydrochloride. It remained the same after the third recrystallization. The yield was only 63 mg. of benzyl-L-cysteine plus 25 mg. which had been expended in radioactivity determinations on the various fractions. The compound was converted to *N*-acetyl-*S*-benzyl-L-cysteine which had the same specific radioactivity as the starting material.

70 mg. of mixed isomers of benzylcysteine were recovered from the original mother liquors. This material was racemized and converted to *N*-acetyl-*S*-benzyl-DL-cysteine by the method of Wood and du Vigneaud (1). The specific activity of this DL preparation was 3.2 times that of the L obtained in the resolution.

TABLE III

Preparation of Radioactive Benzyl-D-homocysteine and N-Acetyl-S-benzyl-D-homocysteine from Radioactive Benzyl-DL-homocysteine

Compound recrystallized	Fraction No.	Weight of sample used	Volume	Recovery	Specific radioactivity of sulfur	Estimated loss of L isomer
		mg.	*ml.*	*mg.*	*counts per sec. per γ*	*per cent*
Benzyl-D-homocysteine	1	110*	10	91	1.84	32
	2	88	10	81	1.43	70
	3	40	10	25	1.10	100
N-Acetyl-*S*-benzyl-D-homocysteine	1	30†		30	1.14	
	2	24	10‡	4	1.05	

* This sample consisted of 4.94 mg. each of radioactive benzyl-L-homocysteine and benzyl-D-homocysteine in DL mixture diluted with 100 mg. of unlabeled benzyl-D-homocysteine, a dilution factor of 21.04. The specific radioactivity before dilution was 22.1 counts per second per microgram of S. The solubility of benzyl-D-homocysteine was estimated at 0.5 mg. per ml. and that of benzyl-DL-homocysteine at 1 mg. per ml.

† 30 mg. of benzyl-D-homocysteine.

‡ 10 ml. of a 50 per cent ethanol and water mixture.

Preparation of Radioactive Benzyl-DL-homocysteine—Benzyl mercaptan was prepared from radioactive sulfur (1 mM) as previously described (7). The solution was dried with sodium sulfate and the solvent was distilled. To the residue of radioactive benzyl mercaptan was added 0.07 ml. of unlabeled benzyl mercaptan in 5 ml. of 0.48 M sodium ethoxide, making a total of 1.1 mM of benzyl mercaptide. To this solution was added a suspension of 240 mg. of pure 3,6-bis(β-chloroethyl)-2,5-diketopiperazine[1] in 5 ml. of absolute ethanol. The suspension was heated under a reflux for 1 hour. The solvent was distilled and 10 ml. of 9 M hydrochloric acid were added to the nearly dry residue. The suspension was heated under a reflux at 110–120° for 7 hours. The solution was then allowed to cool to room temperature, filtered with charcoal, and concentrated nearly to dryness *in vacuo*. 5 ml. of distilled water were added and the solution was again concentrated *in vacuo*. The residue was taken up in 10 ml. of distilled water and neutralized with concentrated ammonium hydroxide. The precipitate was collected, washed successively with distilled water, cold absolute ethanol, and cold ether. The benzyl-DL-homocysteine weighed 55.3 mg., which represented a yield of 25 per cent of the theoretical amount, and melted at 240–243° with preliminary darkening. On a trial run the product was acetylated according to the procedure of du Vigneaud and Irish (8). *N*-Acetyl-*S*-benzyl-DL-homocysteine has a melting point of 115°.

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[1] We are indebted to Dr. E. E. Howe of Merck and Company, Inc., for a generous amount of 3,6-bis(β-chloroethyl)-2,5-diketopiperazine.

Radioactive Benzyl-D-homocysteine—To 9.87 mg. of radioactive benzyl-DL-homocysteine were added 100 mg. of unlabeled benzyl-D-homocysteine.[2] The mixture was dissolved in 5 ml. of N hydrochloric acid and reprecipitated by the addition of 5 ml. of N sodium hydroxide. The pH was adjusted to 7 with a few drops of 0.1 N hydrochloric acid and the mixture was placed in an ice bath for several hours. The precipitate was collected and washed with cold absolute ethanol and cold ether. After it had been dried over phosphorus pentoxide *in vacuo*, the precipitate weighed 91 mg. Table III shows the results of radioactivity determinations on this and subsequent recrystallizations. The acetyl derivative was prepared with no alteration of the specific radioactivity of the sulfur.

DISCUSSION

The method appears to be generally applicable to the resolution of radioactive α-amino acids or other radioactive racemic compounds.[3] Both isomers should be obtainable from the same radioactive racemate. After the first crystalline isomer, for example the L, has been separated, the mother liquor contains the labeled D isomer with its original specific radioactivity and L isomer of a specific radioactivity which is equal to that of the isolated solid material. A labeled D preparation may be crystallized from the mother liquor after the solution has been supersaturated with unlabeled D isomer. In order to follow its purification by radioactivity measurements, it is apparent that a dilution factor must be selected which is significantly greater than the one which was used in the isolation of the

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[2] Benzyl-D-homocysteine was kindly supplied by Dr. Vincent du Vigneaud.

[3] At least one other instance has been reported by Weinhouse and Millington (9) who diluted radioactive DL-tyrosine with unlabeled L-tyrosine and recrystallized the product. However, no evidence was offered to establish the degree of completeness of the resolution.

L isomer. The presence of contaminating radioactive L molecules in the D preparation may then be detected on the basis of the differences in their respective specific radioactivities. It should be noted that saturating the solution of the racemate with both enantiomorphs in turn, during the resolution, will increase the total amount of racemate present. In order to prevent separation of DL crystals the second isomer should be crystallized from an appropriately increased volume of solution.

As an alternative to the separation of the second isomer, the mixture of D and L isomers remaining in the mother liquor may be racemized. A second isotopic dilution with more unlabeled enantiomorph may be carried out. This will permit the crystallization of additional labeled isomer of somewhat lower specific radioactivity.

In applying the method a solvent should be so chosen as to prevent, as far as possible, interaction between the various molecular species in the solution or solid phase. It may be necessary, as shown in the experimental part, to recrystallize more than once in order to attain constant specific radioactivity in succeeding fractions. However, this is not always sufficient to prove completeness of the resolution of labeled molecules. It is becoming increasingly apparent that recrystallization does not always eliminate very minute amounts of radioactive impurities (10, 11). If the compound in question, however, can be converted to a derivative without change in the specific radioactivity, as illustrated in the experimental part, the concept of purity established by recrystallization to constant radioactivity will be greatly extended.

An additional check on the completeness of the resolution of isomers may be obtained by comparing the specific radioactivity of the final product with that of the unresolved material. The final specific activity multiplied by the weight dilution factor can never be greater than the original activity. If it is less, the original DL substance was likely contaminated with other radioactive compounds. In such instances the procedure presented here accomplishes both the resolution of radioactive molecules and the elimination of radioactive impurities. The radioactive racemate should be synthesized with a high specific activity. This will permit the use of large dilution factors in the resolution and thus keep the loss of radioactive molecules in the mother liquors at a minimum.

SUMMARY

A procedure for the resolution of radioactive racemates based on isotope dilution has been applied to the preparation of radioactive L-cystine and radioactive D-methionine.

Radioactive benzyl-DL-cysteine diluted with unlabeled benzyl-L-cysteine was resolved to radioactive benzyl-L-cysteine by fractional recrystalliza-

tions. The progress of the resolution was followed by measurements of the specific radioactivity in successive fractions. Crystallization to constant specific radioactivity and conversion of the last fraction to optically pure, radioactive L-cystine or radioactive *N*-acetyl-*S*-benzyl-L-cysteine with no change in the specific radioactivity indicated that the resolution was complete.

The resolution of radioactive benzyl-DL-homocysteine, an intermediate in the synthesis of methionine, to radioactive benzyl-D-homocysteine was accomplished by the same method. Conversion of the last fraction to the acetyl derivative and recrystallization of the derivative without change in the specific radioactivity indicated that complete resolution to benzyl-D-homocysteine had been achieved.

The synthesis of radioactive benzyl-DL-homocysteine on a micro scale has been reported.

Conditions for the resolution of other radioactive asymmetric compounds by the isotope dilution method have been outlined, and the criteria which were employed in ascertaining the extent of the resolution have been discussed.

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STUDIES ON CHOLINESTERASE

VI. KINETICS OF THE INHIBITION OF ACETYLCHOLINE ESTERASE*

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Enzyme inhibitors have long been used in the analysis of cellular function for separating different steps in complex chemical reactions and for studying the effect of blocking these reactions in the intact cell. Inhibitors of choline ester-splitting enzymes, especially the alkaloids prostigmine and eserine, have attracted the interest of investigators in view of the physiological rôle of acetylcholine. In contrast to the inhibitors previously known, some powerful agents recently developed inactivate the enzymes irreversibly. This property has made it possible to approach many new problems for which the compounds previously available were not appropriate. Particularly, the diisopropyl fluorophosphate (DFP), in the presence of which the irreversible enzyme inactivation is a relatively slow process, has been an excellent tool for testing the essentiality of acetylcholine-hydrolyzing enzymes in nerve conduction (1–3).

In addition, the new compounds offer an opportunity for studying whether the signs of toxicity must be attributed exclusively to interaction with the enzymes. As such an interaction depends on many factors, *in vitro* and still more *in vivo*, it is imperative to study the kinetics of the inhibition of specific esterases, the inactivation of which appears to be primarily responsible for the toxic symptoms (4, 5). The properties of the esterases of conductive tissue and erythrocytes are distinctly different from other choline ester-splitting enzymes (6, 7). The term acetylcholine esterase (ACh-esterase) has been proposed (8) for this type of esterases.

In recent studies on the kinetics of the inhibition of ACh-esterase some differences between DFP and the alkaloids have been described (9, 10). As before, the ACh-esterase used was exclusively the highly purified preparation obtained from the electric tissue of *Electrophorus electricus*.

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Methods

The enzyme preparation, obtained as previously described (11), had an activity equivalent to 20 gm. of acetylcholine chloride split per hour per ml. of solution. The protein content was 1 mg. per ml. The inhibitors used, prostigmine bromide, eserine sulfate, DFP, and tetraethyl pyrophosphate (TEPP), were freshly prepared for each experiment. They were dissolved in the same buffer solution, containing gelatin, as the enzyme. In the experiments in which the inhibitor effects were tested with various substrate concentrations, the final molar concentrations of acetylcholine were 1.1×10^{-1}, 3.3×10^{-2}, 1.1×10^{-2}, 3.3×10^{-3}, 1.1×10^{-3}, 3.3×10^{-4}. In most of the other experiments, the acetylcholine concentration was 3.3×10^{-3} M, which is close to the optimum. The incubation of the enzyme with TEPP at 10°, the subsequent dilution, and the manometric determinations of the enzyme activity with the Warburg method were carried out as described in a preceding study (10). The same procedure was also used in the experiments in which the protective action of prostigmine and eserine against DFP and TEPP was tested. The course of the enzymatic hydrolysis of acetylcholine in the presence of inhibitors and in various substrate concentrations was determined manometrically at 23–24° in the modification described (7).

Results

In Paper V (10) only the enzyme inhibition by DFP was analyzed and compared with the kinetics of the inhibition by the alkaloids prostigmine and eserine. The present experiments include tetraethyl pyrophosphate, found by DuBois and Mangun (12) to be a very potent inhibitor of choline ester-splitting enzymes.

Irreversibility of TEPP Effect—The action of this compound on ACh-esterase is almost immediately irreversible (Table I). This has been tested with the dilution method described for the DFP action. The concentrated enzyme solution was incubated with TEPP at 10° for varying periods of time, diluted 5000 times, and tested for enzymatic activity. This dilution brought the concentration of TEPP into a completely ineffective range. When the enzyme is incubated with DFP under similar conditions, the activity may at first be completely restored, but gradually the reactivation becomes less complete. At 10° it may take 2 to 3 hours until the process becomes completely irreversible. In contrast, the TEPP effect appears to be complete almost immediately. After only 2 minutes incubation at low temperature, no reactivation was obtained by dilution, and after 120 minutes incubation the effect for the various concentrations used was about the same as after 2 minutes. The concentration of TEPP required for a 50 per cent inhibition is much lower than in the case of DFP, the inhibitory effect thus being apparently not only faster but also stronger.

Inhibition by TEPP As Function of Enzyme Concentration—Although the action of TEPP is much stronger than that of DFP, a considerable excess of inhibitor over enzyme concentration is necessary for producing 50 per cent inhibition. In the most concentrated solution, the enzyme concentration during incubation (Table II) is about 4 × 10^{-8} M, estimated on the assumption of a molecular weight of about 3 million. This figure is calculated on the basis of the sedimentation rate in the analytical run in which only one component was present (11). In this concentration, TEPP in 1.4 × 10^{-6} M concentration produces 50 per cent inhibition after 2 minutes incubation. The inhibitor is thus about 35 times in excess of the enzyme. With increasing dilution of the enzyme, the excess of inhibitor required for obtaining 50 per cent inhibition increases. In the greatest dilution tested, the excess is close to 3000 times. In the case of DFP, the excess in the highest concentration used was about the same as with TEPP. In the greatest dilution, however, the excess was

TABLE I

Irreversible Inactivation of ACh-esterase by TEPP

The enzyme was incubated with TEPP for varying periods of time at 10°. Subsequently, the solution was diluted and the enzyme activity determined. b_{30} = enzymatic hydrolysis expressed in μl. of CO_2 evolved per 30 minutes.

TEPP concentration	Incubation period	b_{30}	Per cent inhibition
M × 10^6	*min.*		
Control		195	
0.69	60	148	24
1.38	15	98	50
1.38	30	115	41
1.38	60	92	53
2.76	60	67	66
Control		199	
0.69	60	146	27
0.69	120	155	22
1.38	2	108	46
1.38	4	107	46
1.38	8	107	46
1.38	60	106	47
1.38	120	100	50
2.76	60	39	80
2.76	120	14	93

more than 100,000 times. Much longer incubation periods were used in the case of DFP, and the results are therefore not comparable.

Course of Inhibitor Reactions—The course of the enzymatic hydrolysis of acetylcholine in the presence of the two types of inhibitors (reversible and irreversible) has been studied for various inhibitor concentrations (Figs. 1 to 4). In one series of experiments, the enzyme was incubated with the inhibitor prior to the addition of acetylcholine (*A*); in a second series, inhibitor and substrate came into contact with the enzyme simultaneously (*B*). The optimum acetylcholine concentration was used (3.3×10^{-3} M).

TABLE II

Inhibition of ACh-esterase by TEPP As Function of Varying Enzyme Concentrations

The enzyme in varying dilutions was incubated for a period of 5 minutes at 10° with different inhibitor concentrations. Subsequently, the enzyme was diluted to a concentration 1:5000 of the original solution and the activity determined. The experiments with the lowest dilution, 1:5000, were incubated directly in the Warburg vessel. b_{30} as in Table I.

Enzyme concentration	Inhibitor concentration	Experiment 1		Experiment 2	
		b_{30}	Per cent inhibition	b_{30}	Per cent inhibition
	M				
Undiluted	Control	195		199	
	6.9×10^{-7}	148	24	146	27
	13.8×10^{-7}	92	53	106	47
	27.6×10^{-7}	67	66	39	82
10 × dilution	Control	186		184	
	1.7×10^{-7}	102	46	84	54
	3.45×10^{-7}	44	76	40	78
	6.9×10^{-7}	0	100		
100 × "	Control	186		183	
	2.15×10^{-8}			133	22
	4.3×10^{-8}	92	51	102	44
	8.6×10^{-8}	47	75	55	70
	17.2×10^{-8}	17	91	18	90
1000 × "	Control	207			
	4.3×10^{-8}	75	68		
	8.6×10^{-8}	23	89		
5000 × "	Control	200		207	
	0.26×10^{-8}	188	6		
	0.7×10^{-8}	154	23		
	2.3×10^{-8}	100	50	103	50
	4.6×10^{-8}			63	70

Prostigmine—When the enzyme is incubated with various concentrations of prostigmine for 70 minutes before the addition of acetylcholine, it is 10 to 25 minutes until equilibrium is reached, the time depending upon the inhibitor concentration used (Fig. 1, *A*). During this period, the inhibition is stronger. Without incubation, the period of time required for reaching the equilibrium is about the same, but during this period the inhibition is less strong than in equilibrium. In one case, the inhibitor reacts with the enzyme before the substrate is present; in the other case substrate and inhibitor compete for the enzyme. It is therefore not surprising that the initial reaction velocities in the two types of experiments are different. However, after equilibrium the reaction velocities are the same for the same concentration of prostigmine. This is consistent with previous observations that prostigmine inactivates ACh-esterase completely reversibly.

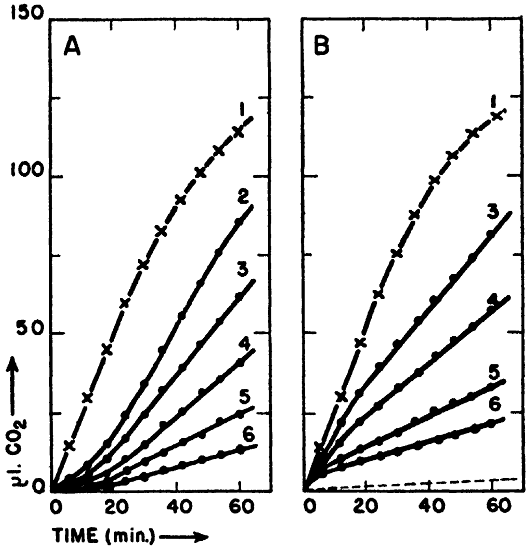

FIG. 1. The course of hydrolysis of acetylcholine in the presence of various concentrations of prostigmine. *A*, enzyme incubated 70 minutes with the inhibitor before the addition of acetylcholine (final concentration 3.3×10^{-3} M). *B*, inhibitor and acetylcholine simultaneously mixed with the enzyme. The dotted line refers to non-enzymatic hydrolysis. Curve 1 is the control, Curves 2 to 6, the hydrolysis in the presence of prostigmine in 6.25, 12.5, 25, 50, and 100×10^{-7} M concentration (during incubation 5 times higher).

The decrease of the reaction velocity at the end of the control experiment is due to the low concentration to which the acetylcholine has been reduced by the hydrolysis. The total amount of CO_2 which can be evolved from the acetylcholine solution used is 148 μl.

Eserine behaves similarly to prostigmine. The period of time until the equilibrium is reached depends upon the concentration of the inhibitor. In equilibrium, the inhibition is the same in the experiments with and without incubation (Fig. 2). As shown by other authors, the reaction between eserine and ACh-esterase is completely reversible.

In cases in which the reactions were followed for a longer time (2 hours and more), the inhibition was slower. This may be due to inactivation of eserine, observed by Ellis and coworkers (13) in experiments with serum cholinesterase.

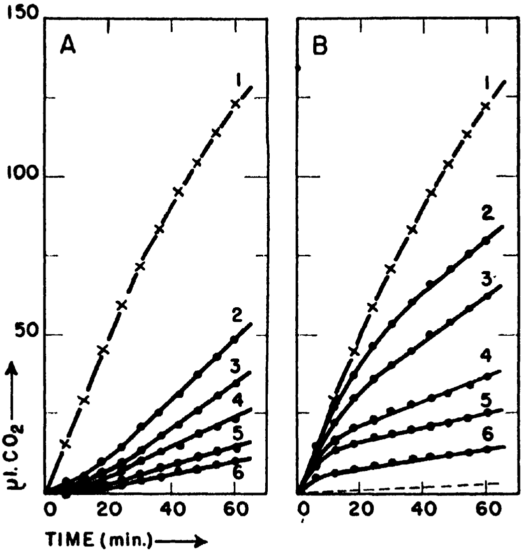

FIG. 2. The course of hydrolysis of acetylcholine in the presence of various concentrations of eserine. *A*, enzyme incubated 60 minutes with the inhibitor before the addition of acetylcholine (final concentration 3.3×10^{-3} M). *B*, inhibitor and acetylcholine simultaneously mixed with the enzyme. Curve 1 is the control, Curves 2 to 6, the hydrolysis in the presence of eserine in 6, 12, 24, 48, and 96×10^{-7} M concentration (during incubation 5 times higher).

DFP—As pointed out, the fundamental difference between the inhibition of ACh-esterase by DFP and that by prostigmine and eserine is the reversible nature of the latter inhibition. When the enzyme was incubated with DFP for 60 minutes at 1.25×10^{-6} M, it lost 50 per cent of its activity (Fig. 3). When the enzyme was mixed simultaneously with acetylcholine and DFP, without incubation, much higher inhibitor concentrations were necessary for obtaining 50 per cent inhibition. At a concentration of 10^{-5} M DFP, no inhibition at all was observed during the first 40 minutes. After that period, the activity decreased slowly. With 10^{-4} M DFP, the inhibition started earlier but no real equilibrium was reached. Only when the DFP concentration was as high as 10^{-4} M was the inhibition nearly complete.

The results suggest that the acetylcholine protects the enzyme against DFP. However, the acetylcholine concentration (3.3×10^{-4} M) is 30 times as high as the inhibitor concentration in the first experiment (10^{-5} M DFP), and 3 times as high in the second experiment. On the other hand, in the experiment in which the inhibition of the hydrolysis was nearly complete, the concentration of DFP was 3 times as high as that of acetylcholine.

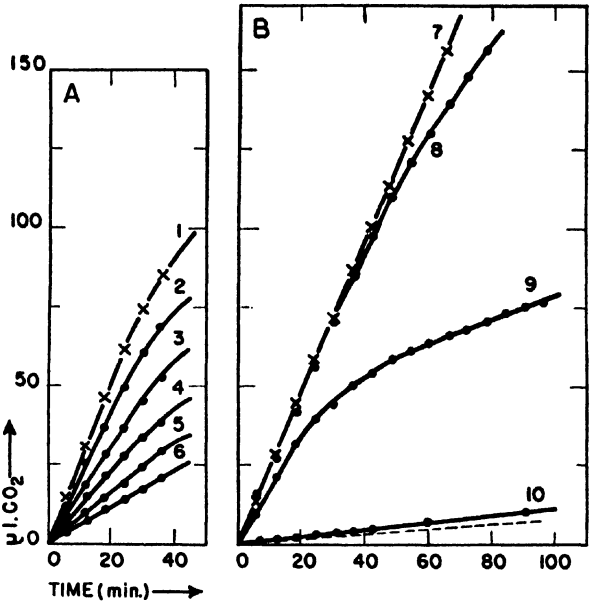

FIG. 3. The course of hydrolysis of acetylcholine in the presence of various concentrations of DFP. *A* and *B*, as in Fig. 2, but acetylcholine concentration in $B = 1 \times 10^{-2}$ M. Curves 1 and 7 are the controls, Curves 2 to 6, the hydrolysis in the presence of DFP in 1.25, 2.5, 5.0, 8.75, and 10×10^{-7} M concentration, Curves 8 to 10 in 1.5, 10, and 100×10^{-5} M concentration.

A much stronger protective action may be obtained with prostigmine. This compound may protect the enzyme against DFP if present in the same concentration (Table III[1]). The enzyme was incubated with prostigmine for 20 minutes at 10° prior to the addition of DFP. The activity of the enzyme after 150 minutes incubation with DFP may be restored partly or nearly completely by dilution (5000 times). Without prior incubation with prostigmine, 50 per cent of the enzyme was irreversibly inactivated. With prostigmine at half the concentration of DFP, the protection was half as strong. At room temperature the protective effect of prostigmine seems less complete. Incubation with eserine protects the enzyme much less against subsequently added DFP than does incubation with prostigmine. This finding is in agreement with the observations of Koelle (14) that eserine has a protecting effect against the action of DFP in brain homogenates.

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[1] These experiments were carried out in collaboration with Dr. M. A. Rothenberg.

TABLE III

Protective Effect of Prostigmine against Action of DFP on ACh-esterase

The enzyme solution was incubated for 150 minutes with DFP (final concentration 1.6×10^{-6} M), then diluted about 5000 times, and the activity was measured. Prostigmine (or eserine) was added to the solution 20 minutes prior to DFP. The compounds were added always in 0.1 ml. to 0.1 ml. of enzyme solution; the total volume was in all cases 0.3 ml. The final concentration of prostigmine (or eserine) was the same as that of DFP (1.6×10^{-6} M) except in one case, 3.2×10^{-6} M (2 ×) and in two cases 0.8×10^{-6} M (½ ×). b_{30} = enzymatic hydrolysis expressed in μl. of CO_2 per 30 minutes. Experiments 1 to 3, at 10°; Experiment 4, at 23°.

Compound	b_{30}	Per cent inhibition
Control	131	
DFP	65	50.5
Prostigmine + DFP	131	0
"	145	0
Eserine + DFP	85	35.0
"	137	0
Control	115	
DFP	60	48.0
Prostigmine + DFP	92	20.0
(½ ×) Prostigmine + DFP	78	32.0
Prostigmine	108	6.0
Control	238	
DFP	118	50.5
(2 ×) Prostigmine + DFP	218	8.5
Prostigmine + DFP	224	6.0
(½ ×) Prostigmine + DFP	178	25.0
Control	210	
DFP	55	74.0
Prostigmine + DFP	131	37.5
"	205	2.5
Eserine + DFP	77	63.5
"	203	3.5

TEPP inhibits the enzyme activity in a manner similar to DFP, but much more strongly. On incubation for 60 minutes with 8×10^{-9} M TEPP, the inhibition is 50 per cent (Fig. 4, *A*). In experiments without incubation, the acetylcholine seems to protect the enzyme against the action of TEPP and no equilibrium is reached between inhibitor, substrate, and enzyme. A weak effect is obtained with a concentration of 3.5×10^{-7} M, which is 125 times higher than that producing 50 per cent inhibition after incubation. The inhibitory effect progresses with time.

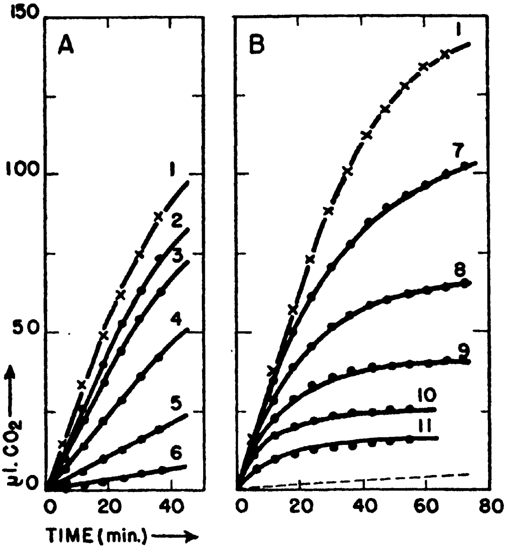

FIG. 4. The course of hydrolysis of acetylcholine in the presence of various concentrations of TEPP. *A* and *B*, as in Fig. 2. Curve 1 is the control, Curves 2 to 11, the hydrolysis in the presence of TEPP in 0.7, 1.4, 2.8, 5.6, 11.2, 87.5, 175, 350, 700, and 1400 $\times 10^{-9}$ M concentration.

The enzyme was also incubated with prostigmine or eserine prior to the addition of TEPP and the activity then tested to subsequent dilution. No protective action was observed if the two types of inhibitors were added in the same concentration.

Inhibition As Function of Inhibitor Concentration—The inhibition as a function of inhibitor concentration may be analyzed by plotting v/v' against the concentration of the inhibitor, v being the reaction velocity in the absence of the inhibitor I, v' in its presence. In the case of competitive inhibition and constant concentrations of enzyme and substrate, a straight line is thereby obtained, according to Equation 1.

$$\frac{v}{v'} = 1 + [I]\frac{K_S}{K_I([S]+K_S)} \qquad (1)$$

$[S]$ and $[I]$ are the concentrations of substrate and inhibitor respectively; K_S and K_I represent the dissociation constants of the complexes between enzyme and substrate and enzyme and inhibitor respectively. The intercept of the straight line on the ordinate (v/v') is 1. From the slope of the line (*i.e.*, $K_S/K_I([S] + K_S)$ and known values of $[S]$ and K_S, K_I can be calculated. This method has been applied by Augustinsson (15) with satisfactory results for the analysis of the action of choline on various esterase systems and has therefore been used in the present studies.

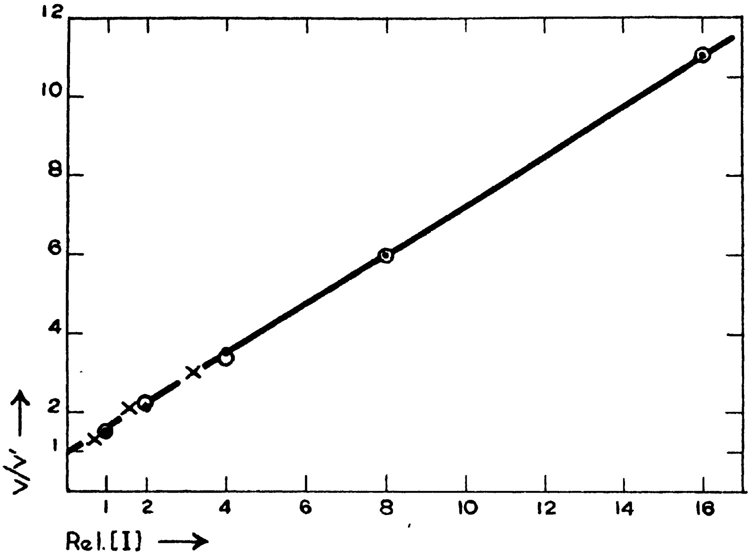

FIG. 5. Inhibition of ACh-esterase by prostigmine as function of inhibitor concentration. v = velocity in absence, v' in the presence of inhibitor, expressed in μl. of CO_2 evolved in 30 minutes (b_{30}). Acetylcholine concentration, 3.3×10^{-3} M; inhibitor concentration, relative inhibitor concentration (Rel.$[I]$), 1 = 6.25×10^{-7} M. ●, without incubation; ○, with incubation; ×, values obtained for the above concentration in the experiments with varying substrate concentrations (Fig. 9). $K_I = 1.6 \times 10^{-7}$.

Prostigmine and Eserine—Applying this method of analysis for the action of prostigmine and eserine on ACh-esterase results in straight lines (Figs. 5 and 6). The data are the same in experiments with and without incubation. This indicates that the two alkaloids inhibit the esterase activity competitively; *i.e.*, that they react reversibly with the same active center of the enzyme as does acetylcholine.

The affinity of the enzyme is 2.6 times higher for eserine than for prostigmine. The dissociation constants for the enzyme inhibitor complexes are 1.6×10^{-7} and 6.1×10^{-8} respectively, calculated according to Equation 1.

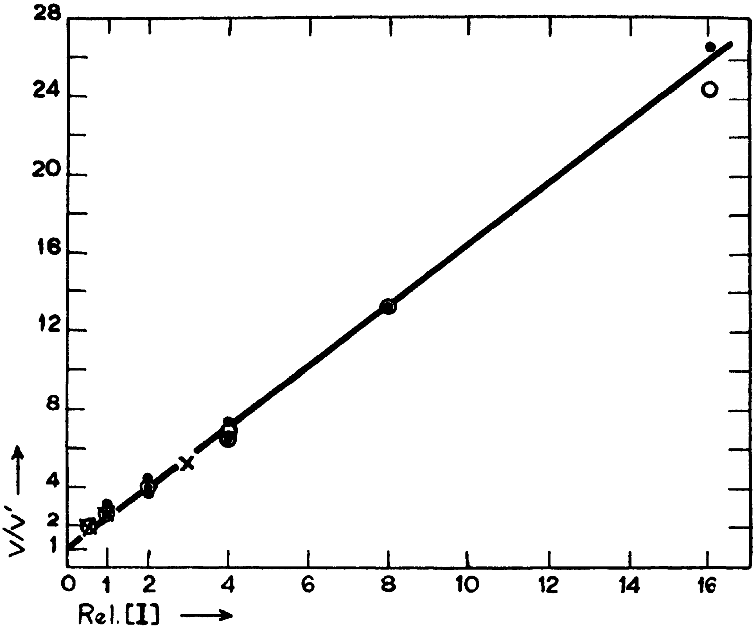

FIG. 6. Inhibition of ACh-esterase by eserine as function of inhibitor concentration. Description as in Fig. 5. Relative inhibitor concentration (Rel. [*I*]), 1 = 6.0×10^{-7} M. $K_I = 6.1 \times 10^{8}$.

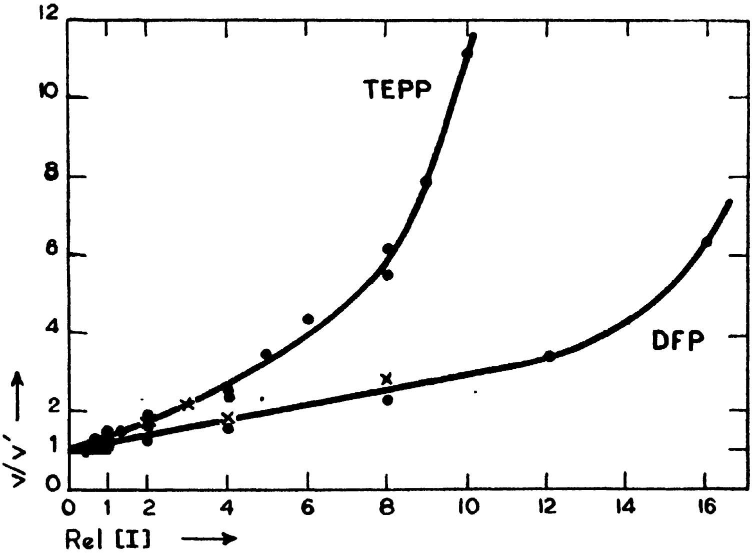

FIG. 7. Inhibition of ACh-esterase by DFP and TEPP respectively as a function of inhibitor concentration. Description as in Fig. 5. Relative inhibitor concentration (Rel. [*I*]), 1 = 2.5×10^{-7} M DFP and 2.8×10^{-9} M TEPP respectively.

DFP and TEPP—Both DFP and TEPP behave differently from the alkaloids. By plotting the degree of inhibition expressed by v/v' against the inhibitor concentration, straight lines are not obtained (Fig. 7). With increasing DFP and TEPP concentrations, the inhibition increases more rapidly than is expected in a competitive inhibition. The curves in Fig. 7 are based on the data obtained when the enzyme had been incubated for 1 hour with the inhibitors.

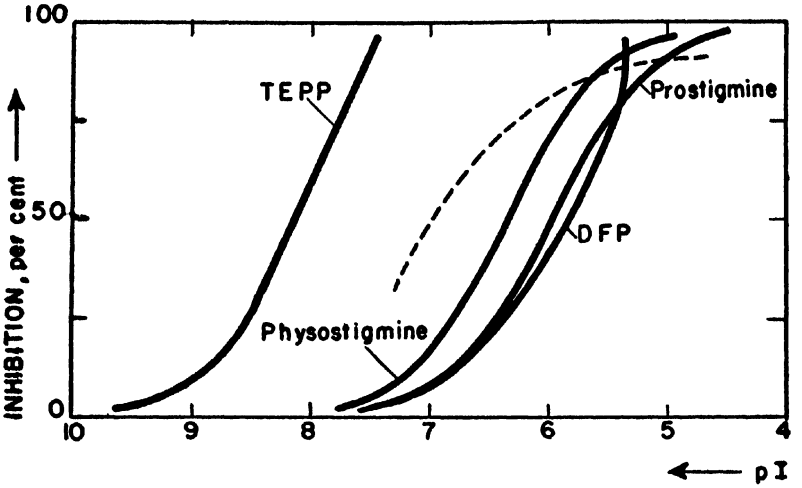

FIG. 8. Per cent inhibition of ACh-esterase by prostigmine, eserine, DFP, and TEPP, respectively, as function of the negative log of the molar concentration of the inhibitors (pI). The dotted line refers to the effects obtained with prostigmine and eserine when measured immediately after addition of acetylcholine before equilibrium is reached.

A picture of the relative strength of the four inhibitors studied is obtained if the per cent inhibition is plotted against pI, the negative log of the molar concentration of inhibitor (Fig. 8). The dotted line indicates the values obtained if the activity of the two alkaloids is measured during a period of 20 to 30 minutes after addition of acetylcholine following incubation. As pointed out before, during that period the equilibrium has not yet been reached and these values, used in the preceding study (10), indicate a stronger inhibition than that obtained in equilibrium.

Inhibition As Function of Substrate Concentration—The inhibition of ACh-esterase at various substrate concentrations by the four inhibitors is shown in Figs. 9 to 12. The enzyme was incubated for 60 minutes with the inhibitor before determining the activity.

In presence of prostigmine, the optimum substrate concentration is changed to a higher concentration in the presence of the drug (Fig. 9).

With increasing inhibitor concentration, this shift becomes increasingly stronger. The degree of inhibition for a given inhibitor concentration therefore varies greatly with the substrate concentration. At 3.3 × 10^{-2} M acetylcholine, the enzyme is inhibited 10 per cent by 2 × 10^{-6} M prostigmine; at 3.3 × 10^{-3} M acetylcholine, the inhibition by the same concentration of prostigmine is as high as 88 per cent.

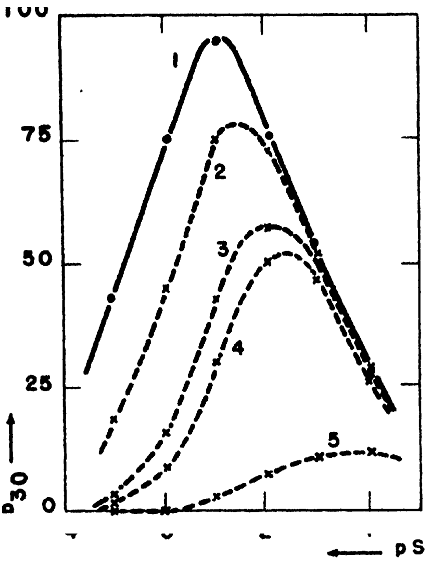

FIG. 9

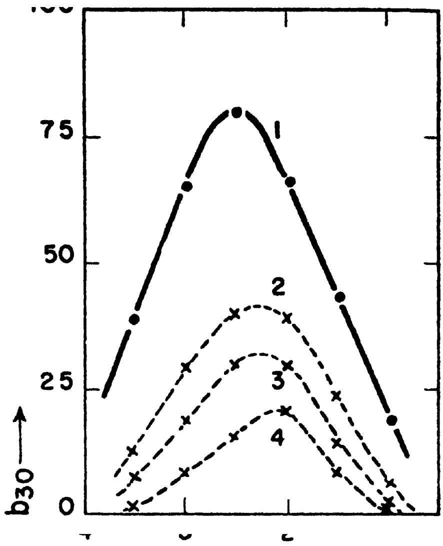

FIG. 10

FIG. 9. Activity-pS curves for the enzymatic hydrolysis of acetylcholine by ACh-esterase in the presence of various concentrations of prostigmine. Curve 1 is the control, Curves 2 to 5, the hydrolysis in the presence of prostigmine in 0.4, 1, 2, and 10 × 10^{-6} M concentration.

FIG. 10. Activity-pS curves for the enzymatic hydrolysis of acetylcholine by ACh-esterase in the presence of various concentrations of eserine. Curve 1 is the control, Curves 2 to 4, the hydrolysis in the presence of eserine in 3, 6, and 18 × 10^{-7} M concentration.

Eserine—According to the results demonstrated in Fig. 6, eserine, like prostigmine, inhibits acetylcholine competitively. However, the optimum substrate concentration is only slightly changed in the presence of low eserine concentrations. The shift becomes more pronounced at relatively high eserine concentrations. In that case, the optimum substrate concentration is definitely higher than in the absence of the inhibitor (Fig. 10). This agrees with the finding that in the presence of 3.6 × 10^{-6} M eserine the optimum acetylcholine concentration (pS) for the erythrocyte esterase of horse blood is 1.5, whereas it is 2.6 in the absence of the inhibitor (15).

DFP and TEPP—In the case of the irreversible inhibitors, the inhibition is independent of the substrate concentration. This is illustrated in Figs. 11 and 12. At a concentration of 5×10^{-7} M DFP, for example, the enzyme is inhibited to about 70 per cent at all substrate concentrations.

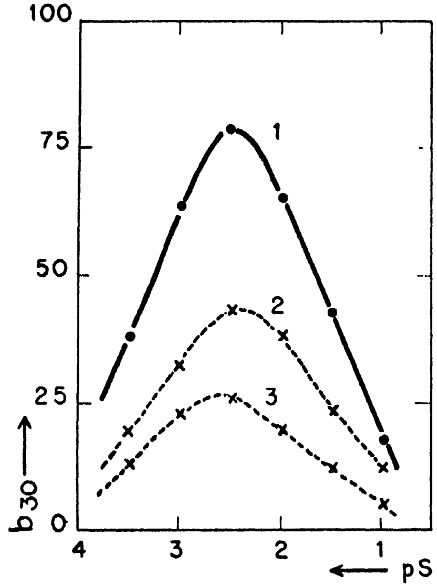

FIG. 11

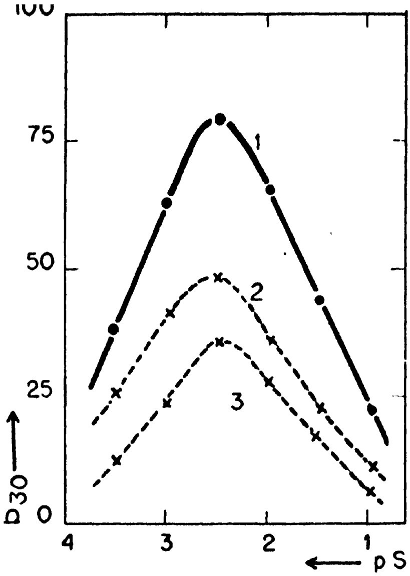

FIG. 12

FIG. 11. Activity-p*S* curves for the enzymatic hydrolysis of acetylcholine by ACh-esterase in the presence of DFP. Enzyme incubated 60 minutes with the inhibitor before addition of acetylcholine. Curve 1 is the control, Curves 2 and 3, the hydrolysis in the presence of 1 and 2×10^{-6} M concentration of DFP (during incubation; during determination, the concentration was 5 times lower).

FIG. 12. Activity-p*S* curves for the enzymatic hydrolysis of acetylcholine by ACh-esterase in the presence of TEPP. Enzyme incubated 60 minutes with the inhibitor before addition of acetylcholine. Curve 1 is the control, Curves 2 and 3, the hydrolysis in the presence of 5.6 and 8.4×10^{-9} M concentration of TEPP (during incubation; during determination, the concentration was 5 times lower).

DISCUSSION

Several new differences between the various inhibitors of choline ester-splitting enzymes have been revealed in addition to those described previously (10). The sequence in which inhibitor and substrate come in contact with the enzyme affects the course of hydrolysis in the case of the reversible inhibition, the alkaloids prostigmine and eserine, in a way different from that found in the case of DFP and TEPP. Whether the enzyme is first incubated with alkaloids or whether substrate and inhibitor are added simultaneously, the degree of inhibition is the same after equilibrium has been reached. Before the attainment of an equilibrium, a period which lasts 10 to 25 minutes, depending upon the concentration used, the inhibition is stronger than during equilibrium when the enzyme has been incubated with the inhibitor. Since both compounds are competitive inhibitors of the enzyme and the formation of the enzyme complex in both cases is completely reversible, it should be expected that the equilibrium is the same irrespective of incubation. The substrate concentration is much higher than the inhibitor concentration and this may explain why, in the case of simultaneous addition, the inhibition is less strong in the beginning than later, whereas if the enzyme has formed a complex with the inhibitor before the substrate is added, the inhibition is stronger in the beginning. When v/v', the ratio of the velocity of the hydrolysis in absence and presence of the inhibitor, is plotted against inhibitor concentration, the same straight line is obtained with or without incubation with both alkaloids. But the inhibitory effect of eserine is about 2.6 times stronger than that of prostigmine.

In the case of irreversible inhibition, the degree of inhibition depends upon the time of incubation, as was previously demonstrated for DFP (10). Therefore, no equilibrium is attained, and if v/v' is plotted against inhibitor concentration, the line is not straight. The same is true for TEPP, which, like DFP, inactivates the enzyme irreversibly, though more rapidly. If acetylcholine is added before the inhibitor, much higher concentrations of the latter become necessary for obtaining the same degree of inhibition as that observed after incubation. This protective effect of acetylcholine against the action of these two compounds containing organic phosphorus suggests that these compounds act on the same active center of the enzyme molecule as acetylcholine. This assumption is supported by the observation that prostigmine in the same concentration as the DFP has a very strong protective action. The protective action is obtained with very low concentrations if compared with that of acetylcholine. This may be explained by the high affinity of prostigmine to the enzyme. The affinity of DFP to the enzyme cannot be evaluated since there is no equilibrium, but, as far as one can speak of affinity, it appears to be of a similar order of magnitude as that of prostigmine. Eserine in spite of its stronger inhibitory effect protects the enzyme markedly less against DFP. As long as the exact reaction is unknown, a satisfactory explanation for this difference will be difficult. The lack of any protective action by prostigmine against TEPP if equimolecular concentrations are used is not surprising. It may be explained by a much higher affinity of TEPP to the enzyme, since it inhibits in so much lower concentrations. It would be interesting to test whether a protective action of prostigmine may be obtained in 100 to 1000 times higher concentrations, but the concentration of the available enzyme preparation is too low for such an experiment.

Although it is possible to conclude, on the basis of the data presented, that both types of inhibitors may act on the same center of the enzyme as acetylcholine, but that in one case they form a reversible and in the other an irreversible complex, the underlying chemical reactions are at present unknown. This situation may be analogous to the inhibition of the combination of hemoglobin with oxygen by carbon monoxide and by potassium ferricyanide. Both inhibitors combine with the iron of the prosthetic group, the iron porphyrin. The carbon monoxide effect is easily reversible, whereas the potassium ferricyanide transforms the iron irreversibly.

Study of the competitive inhibition of the enzyme by prostigmine and eserine has revealed another difference between the two alkaloids. Prostigmine causes a marked increase of optimum substrate concentration with increasing inhibitor concentration. It has been demonstrated for erythrocyte and brain esterase that choline exerts a shift of the optimum substrate concentration; the more choline is added, the higher is the optimum substrate concentration and the lower the activity. It is possible that other quaternary ammonium ions may behave similarly and that such compounds may inhibit competitively the formation of the enzyme-substrate complex.

In the case of eserine, the shift of optimum concentration is much less pronounced and is marked only in high concentration of the inhibitor. Therefore, in low (*e.g.*, 10^{-4} M) acetylcholine concentration, both alkaloids inhibit the enzyme at the same concentration to about the same degree; in high (10^{-1} M) acetylcholine concentrations, on the other hand, prostigmine does not affect the enzyme activity at all, whereas eserine has a strong effect. The absence of a shift of optimum in the case of DFP and TEPP may be explained by a gradually lowered activity, since these compounds inhibit the enzyme irreversibly.

SUMMARY

The study of the kinetics of the inhibition of ACh-esterase by two types of inhibitors, reversible and irreversible, has been continued.

TEPP was found to inactivate the enzyme irreversibly, and at 10° almost immediately, in contrast to DFP with which, at this temperature, the irreversible reaction is a relatively slow process. The study of the action of this inhibitor as a function of enzyme concentration has revealed that a considerable excess of inhibitor is necessary, which increases with increasing dilution. The enzymatic hydrolysis of acetylcholine in the

presence of prostigmine and eserine attains an equilibrium which is the same whether or not the enzyme has been incubated with the inhibitor prior to the addition of acetylcholine. No such equilibrium is attained with the irreversible inhibitors, DFP and TEPP. If acetylcholine is added to the enzyme simultaneously with the inhibitor, a protective action is observed, suggesting that all four inhibitors act on the same active center of the enzyme. TEPP is by far the strongest of all four inhibitors tested.

When the inhibitor effects are studied at various substrate concentrations, prostigmine and, to a lesser degree, eserine produce a shift of the optimum substrate concentration. No such shift is observed in the case of the two irreversible inhibitors.

We want to express our thanks to Mrs. Emily Feld-Hedal and to Mrs. M. Augustinsson for their assistance in the experiments.

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PHOSPHOGLUCOMUTASE

I. PURIFICATION AND PROPERTIES*

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Phosphoglucomutase, the enzyme which converts glucose-1-phosphate into glucose-6-phosphate, is a known constituent of various animal tissues and yeast (1, 2). The crude enzyme is activated by Mg^{++}, Mn^{++}, and Co^{++} (2). Najjar (3) has isolated it in crystalline form from rabbit muscle extract by a method consisting of heat denaturation of inert tissue proteins and ammonium sulfate fractionation of the filtrate. The pure preparation had an activity per unit weight 50 times greater than that of the crude muscle extract. The pure enzyme was reported to have very little activity unless it was activated by cysteine. Mg^{++} increased the activity of the enzyme, while Mn^{++} could not be tested, since it forms a complex with cysteine and thereby inhibits phosphoglucomutase activity. The enzyme had maximum activity at pH 7.5 with 0.005 to 0.0025 M Mg^{++} and 0.025 M cysteine.

EXPERIMENTAL

Reagents—The substrate, glucose-1-phosphate, was prepared enzymatically by the method of Hanes (4) and was recrystallized four times from 50 per cent alcohol. Albumin was found essential for complete activation of the enzyme system; crystalline bovine serum albumin, provided through the courtesy of Armour and Company, was employed. As a reducing agent, also essential for activation of the enzyme, sodium sulfite was used; a solution, 0.20 M in concentration, adjusted to pH 7.5 with dilute sulfuric acid, could be preserved at 4° in air-tight bottles for 2 weeks.

Determinations—Inorganic phosphate was determined according to Fiske and Subbarow (5). Colorimetric measurements were carried out at 660 mμ with a Beckman spectrophotometer.

Glucose-1-phosphate was determined by hydrolysis with 1 N sulfuric acid at 100° for 7 minutes and estimation of the equivalent amount of inorganic phosphate liberated. Glucose-6-phosphate is not hydrolyzed under these conditions.

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* This work forms part of a thesis submitted by V. Jagannathan to the Department of Chemistry, Stanford University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Aided by a scholarship from the Government of Madras, India.

Nitrogen was estimated by the method of Johnson (6), the color being read at 490 mμ with a Beckman spectrophotometer. Micro-Kjeldahl determinations were also run on pure enzyme preparations. The values for nitrogen reported in this paper refer only to protein nitrogen.

Estimation of Enzyme Activity—Enzyme solutions were tested for activity as follows except when otherwise stated.

The enzyme solution was diluted with a solution of 0.10 M sodium sulfite containing 1 per cent serum albumin at pH 7.5. To an aliquot of diluted enzyme, magnesium sulfate, sodium sulfite, and serum albumin solutions (all at pH 7.5) were added and made up with distilled water to 0.5 ml. to give final concentrations of sulfate, sulfite, and albumin, respectively, of 10 mM, 20 mM, and 0.4 per cent. After temperature equilibration of this solution at 36°, 0.5 ml. of approximately 32 mM dipotassium glucose-1-phosphate of the same pH and temperature was added. At the end of 10 minutes, 10 ml. of 5 per cent trichloroacetic acid were added and the precipitated proteins were removed by filtering. An aliquot of the filtrate was analyzed for glucose-1-phosphate.

The difference between the acid-labile P before and after enzyme action was a measure of the glucose-1-phosphate converted. The unit of enzyme activity was defined as the amount of the enzyme required to cause a decrease in acid-labile P of 40 γ under the above conditions. Three different dilutions of the enzyme were used for each test, the conversion of substrate being less than 50 per cent in each case.

An aliquot of the enzyme solution was also analyzed for nitrogen and its purity expressed as units of activity per mg. of nitrogen.

Method of Purification—A well fed rabbit was anesthetized by intravenous injection of pentobarbital. It was then perfused with Ringer-Locke solution at 37° to wash out as much blood as possible. The solution was allowed to enter under sufficient hydrostatic pressure through two hypodermic needles inserted into the ear veins of the rabbit and to pass out through the femoral artery. When the animal was dead, the back and thigh muscles were rapidly removed and minced with an equal volume of ice-cold distilled water in a chilled Waring blendor. After being kept at 4° for an hour with occasional stirring, the minced tissue was pressed through cheese-cloth. The residue was similarly reextracted with an equal amount of water.

The combined extracts were adjusted to pH 5.0 with acetic acid and heated in a water bath with stirring so that the temperature rose to 56° in 6 to 7 minutes. After 10 minutes at that temperature the liquid was rapidly cooled and allowed to filter under suction at 4° overnight.

The clear filtrate was then mixed with one-ninth its volume of 1.0 M acetate buffer at pH 5.0 and cooled to 0°. Subsequent operations were carried out at −2° to 0°. Acetone was slowly added with stirring to give a concentration of 40 per cent by volume. After 1 to 2 hours the liquid was centrifuged and the clear supernatant solution treated with more acetone to 50 per cent concentration. The precipitate was collected by centrifugation after 2 hours and dissolved in 0.10 M acetate buffer at pH 5.0.

The solution was reprecipitated as before with 40 per cent acetone. The clear supernatant liquid was then treated with 0.50 M manganous sulfate to give a final concentration of 0.015 M. After 5 to 6 hours the precipitated enzyme was centrifuged and dissolved in the minimum amount of 36 per cent acetone. Insoluble material was removed by centrifugation. The clear solution was then slowly added with stirring to 38 per cent acetone containing 16 mM manganous sulfate and 0.05 M acetate buffer (at pH 5.0 before addition of acetone). The volume of the solution should be such as to give a final concentration of 40 to 60 γ of protein nitrogen per ml. If the protein concentration was too low, the concentration of acetone was slowly raised to 40 to 41 per cent. The solution remained clear initially and a precipitate slowly formed on standing overnight. The precipitate was centrifuged and reprecipitated twice exactly as before.

TABLE I

Purification of Phosphoglucomutase

Enzyme preparation	Volume	Activity	Protein N	Purity
	ml.	*units*	*mg.*	*units per mg. N*
Fresh extract (500 gm. muscle)	1000	410,000	2850	144
Filtrate after heating at 56°	1000	360,000	275	1310
40–50% acetone fraction	30	310,000	69	4500
1st Mn^{++} ppt	20	220,000	40	5500
2nd " "	18	115,000	18	6300
3rd " "	17	62,000	8.6	7200

The final precipitate often contained fragments of crystalline material along with amorphous material, but it cannot be definitely stated whether these crystals were the enzyme or not. Further work on this was not continued.

The results of a typical experiment are given in Table I. The final activity of the enzyme was 7000 to 7100 units per mg. of nitrogen, representing a purification of about 50-fold. Further fractionation failed to increase the purity above this level. Fairly concordant results were obtained by this procedure except when the perfusion of the muscle was unsuccessful. A similar difficulty due to the presence of blood in the muscle was noted by Najjar using a different method of purification (3).

An electrophoretic study was made of the enzyme obtained after the first precipitation with manganous sulfate. One experiment was carried out with the enzyme (4400 units of activity per mg. of nitrogen) in barbiturate buffer at pH 7.7 (24°), ionic strength 0.10, and a temperature of 0.6°. The enzyme was found to be about 62 per cent pure and had a mobility of -1.9×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$; it was negatively charged. A second experiment with the enzyme (5100 units per mg. of nitrogen) in acetate buffer of pH 5.6 (22°), run at 0.05 ionic strength and 0.6°, showed that the enzyme was about 72 per cent pure; it was positively charged and had a mobility of $+1.2 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$. It was unfortunately not possible to study the pure enzyme electrophoretically, but the above results indicate that the enzyme with an activity of 7000 units per mg. of nitrogen should be nearly 100 per cent pure.

Properties of Phosphoglucomutase

Purified enzyme with an activity of 7000 units per mg. of nitrogen was dissolved in 0.10 M acetate buffer at pH 5.0 and dialyzed free of manganous salts against several changes of the same buffer. The dialyzed enzyme was used for the study of its properties.

The enzyme exhibited several unusual properties when tested with various activators (Table II). The pure enzyme showed very little activity in the absence of serum albumin or activating metallic ions. When serum albumin was present and sodium sulfite omitted from the reaction mixture, the enzyme showed the same activity with Mg^{++} or Mn^{++}. The addition of sodium sulfite caused a marked increase in activity with either magnesium or manganous ions, the net increase being somewhat variable with the enzyme sample and the period of storage. But at optimum concentrations of serum albumin and sodium sulfite, the activity of the enzyme in the presence of Mg^{++} was always almost exactly twice the activity shown with Mn^{++}. This effect was observed with enzyme preparations at all levels of purity. When both Mg^{++} and Mn^{++} were added, the activity of the enzyme was the same as that shown with Mn^{++} and was half that obtained with Mg^{++}.

In the presence of 0.02 M cysteine, serum albumin had only a slight activating effect, but the use of cysteine as activator was discontinued, since the comparative study of Mg^{++} and Mn^{++} was not possible with it.

On dilution of the enzyme at room temperature with 1 per cent serum albumin solution and incubation with 20 mM sodium sulfite at 36°, the activity of the enzyme increased slowly with time to a maximum in about

TABLE II

Effect of Activators on Phosphoglucomutase Activity

Serum albumin	$MgSO_4$	$MnSO_4$	$CoSO_4$	$NiSO_4$	Na_2SO_3	Activity
per cent	*mM*	*mM*	*mM*	*mM*	*mM*	*units per ml.*
0	5					5
0.2						12
0.2	5					745
0.2		0.5				760
0.2			0.5			638
0.2				0.5		18
0.2	5				10	2100
0.2		0.5			10	1060
0.2	5	0.5			10	1080

Aliquots of purified enzyme solutions were mixed with activators as indicated, made up to 0.5 ml., and incubated at 36° for 10 minutes; 0.5 ml. of 32 mM substrate was added in each case. The pH of all reactants was 7.5, temperature 36°, time of reaction 10 minutes. The concentrations of activators in the reaction mixture are specified and activities are expressed as units per ml. of enzyme solution.

TABLE III

Effect of Time of Incubation with Sulfite on Enzyme Activity

Enzyme diluted with	Time of incubation	Activity
	min.	*units*
1.0% serum albumin solution	0.5	229
	5	245
	10	262
	20	268
	40	273
	60	287
	80	288
1.0% serum albumin containing 0.1 M Na_2SO_3	1	286
	5	288
	10	286

Equal volumes of enzyme solution (diluted as stated) were made up to 0.5 ml. to contain 20 mM Na_2SO_3, 10 mM $MgSO_4$, and 0.4 per cent serum albumin and were incubated for varying periods of time at 36°; 0.5 ml. of 32 mM substrate was then added to each. pH 7.5, time of reaction 10 minutes.

40 to 60 minutes (Table III). But when the enzyme was diluted at room temperature with 1 per cent serum albumin containing 100 mM sodium sulfite, maximum activity was obtained soon after temperature equili-

bration at 36°, and there was no further increase in activity on prolonged incubation. This is in contrast to the rapid decrease in activity reported by Najjar (3) when the enzyme was incubated with cysteine.

The activity of the enzyme was found to be proportional to the time of reaction until about 60 per cent of substrate was converted. At equilibrium about 5 per cent of the initial glucose-1-phosphate was left in the reaction mixture (*cf.* (3, 7)). The optimum pH for enzyme activity was 7.5 (Table IV), confirming the value reported by other workers (2, 3). The optimum concentration of serum albumin was 0.05 per cent when sodium sulfite was omitted from the reaction mixture and was 0.20 per cent in the presence of sulfite.

TABLE IV

Effect of pH on Enzyme Activity

pH	Activity
	units per ml. enzyme solution
7.7	420
7.5	458
7.3	441
7.0	368
6.5	151
6.0	62
5.0	2

The enzyme was diluted with 0.10 M Na_2SO_3 containing 1 per cent serum albumin at pH 7.5; dilute H_2SO_4 or NaOH was then added to give the desired pH. All other reactants were also at the same pH in each case, and other experimental conditions were as specified in the text for the determination of enzyme activity.

TABLE V

Effect of Mg^{++} Concentration on Enzyme Activity

Concentration of Mg^{++}	Activity	Concentration of Mg^{++}	Activity
mM	*units per ml. enzyme solution*	*mM*	*units per ml. enzyme solution*
0	2	7	228
1	70	10	215
2	146	14	204
5	260	20	147

The experimental conditions were as described in the text, except for the Mg^{++} concentration.

The optimum concentration of Mg^{++} was about 5 mM (Table V), which is higher than the value of 0.5 to 2.5 mM reported by Najjar using cysteine as activator (3).

The optimum concentration of sodium sulfite was 10 to 20 mM (Table VI).

Najjar's procedure for activity determination was published too late for comparison with the above method.

Potassium and calcium ions inhibited the enzyme. Sodium ions had no inhibitory effect, in contrast to the findings of Cori *et al.* (2). The effect of sodium and potassium ions present in the reaction mixture due to the sodium sulfite and the substrate was not evaluated. Arsenate also inhibited the enzyme, while mercuric and cupric ions exerted a pronounced inhibition (Table VII). Cobaltous ions had an activating effect similar to that of manganous ions, while nickelous ions had no effect (Table II).

TABLE VI

Effect of Sulfite Concentration on Enzyme Activity

Concentration of Na_2SO_3	Activity
mM	*units per ml. enzyme solution*
2	185
6	234
10	265
20	252
30	224

The experimental conditions were as described in the text except for the sulfite concentration indicated.

TABLE VII

Inhibition of Enzyme Activity by Ions

Additions	Concentration	Inhibition
	mM	*per cent*
NaCl	50	0
KCl	50	27
$CaCl_2$	25	34
$HgSO_4$	1.0	25
$CuSO_4$	0.1	100
Na_2AsO_4	1.0	53

The experimental conditions were as described in the text except for the additions specified. The figures in the last column refer to the percentage decrease in activity relative to the activity of the enzyme when no inhibitors were added.

The pure enzyme was quite stable at pH 5.0 when kept at 2-5° and lost only 10 per cent of its activity in 2 to 3 weeks. The enzyme was rapidly and irreversibly inactivated at a pH lower than 4 or greater than 9.0.

All preparations of the enzyme contained phosphorus. Prolonged dialysis with stirring against water or buffers at pH 5.0 to 7.0 failed to remove the phosphorus. On the average the enzyme contained 6 to 7 γ of phosphorus per 10,000 units of activity. The possible significance of this constituent of the enzyme is discussed in the following paper (8).

SUMMARY

1. Phosphoglucomutase from rabbit muscle has been purified about 50-fold by heating and fractionation with acetone and manganous sulfate.

2. The enzyme was found to require about 5 mM Mg^{++}, 0.2 per cent serum albumin, and 10 to 20 mM sodium sulfite at pH 7.5 for maximum activity.

3. With Mn^{++} the activity of the enzyme was found to be half of that obtained with Mg^{++}.

4. The enzyme was activated by Co^{++} and inhibited by K^+, Ca^{++}, Hg^{++}, Cu^{++}, and arsenate.

5. The enzyme contains non-dialyzable phosphorus.

The authors wish to thank Miss Hyla Cook for carrying out the electrophoretic measurements. They are also indebted to Armour and Company for contributing the bovine serum albumin used in these studies.

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PHOSPHOGLUCOMUTASE

II. MECHANISM OF ACTION*

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(Received for publication, January 17, 1949)

Doudoroff *et al.* (1) found that when bacterial sucrose phosphorylase was added to a solution of glucose-1-phosphate and radioactive inorganic phosphate a rapid exchange of phosphate between the inorganic and ester fractions occurred. They proposed the following mechanism:

$$\text{Glucose-1-phosphate} + \text{enzyme} \rightleftarrows \text{glucose-enzyme} + \text{phosphate}$$

Schlamowitz and Greenberg (2) made a similar study of the conversion of glucose-1-phosphate to glucose-6-phosphate by phosphoglucomutase in the presence of radioactive inorganic phosphate and glucose labeled with radioactive carbon. Since no exchange took place between the glucose esters and radioactive phosphate or glucose, they concluded that a mechanism similar to that proposed by Doudoroff *et al.* is not operative in this case. They postulated that the reaction takes place through the intermediary formation of a cyclic diester of phosphate.

During the course of work on purification of phosphoglucomutase it was observed that the enzyme invariably contained phosphorus which could not be removed by dialysis. If the phosphorus were part of the enzyme molecule, another mechanism for the action of the enzyme is possible which has not hitherto been considered in enzyme studies. This involves an exchange of reactive groups between the enzyme and substrate. Phosphoglucomutase could combine with glucose-1-phosphate or glucose-6-phosphate to form a double link with glucose through two phosphate bonds. This compound could then be split at either the 1 or 6 position to give the enzyme and the 6 ester or 1 ester as shown in the accompanying diagram.

If such a reaction were to take place, there should be an exchange between the phosphate group of the ester and the phosphate group of the enzyme. The reaction between enzyme and glucose-1-phosphate labeled with P^{32} should lead to a lowering in the specific activity (counts per minute per microgram of P) of the glucose ester, while the enzyme should become radioactive.

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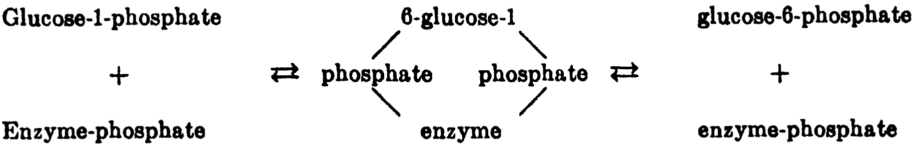
* This work forms part of a thesis submitted by V. Jagannathan to the Department of Chemistry, Stanford University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

† Aided by a scholarship from the Government of Madras, India.

The evidence for such a mechanism is presented in this paper.



Reagents and Measurements

Labeled Glucose-1-phosphate—Since 1 mg. of enzyme contained less than 1 γ of P, it was necessary to prepare a substrate sample with a high count per microgram of P. About 8 mc. of P^{32} (as H_3PO_4) were added to about 0.04 mole of phosphate, which was used for the enzymatic synthesis of glucose-1-phosphate according to the method of Hanes (3). The yield of the dipotassium salt of the ester after three recrystallizations was 0.2 gm. It contained a negligible amount of inorganic phosphate and was 99 to 100 per cent pure. It had an initial radioactivity of 3000 counts per minute per microgram of P.

Phosphoglucomutase—A detailed description of the purification of the enzyme is presented in the preceding paper (4). For the present work enzyme of 70 per cent purity was prepared as follows:

Rabbit muscle extract was adjusted to pH 5.0 with acetic acid, heated at 56° for 10 minutes, and filtered. The filtrate was made 0.1 M with respect to acetate buffer at pH 5.0 and fractionated with acetone at −2°. The fraction obtained between 40 and 50 per cent acetone was similarly reprecipitated twice. The enzyme thus obtained was 70 per cent pure as determined by electrophoretic studies, and it was not further purified owing to insufficient yields of the pure enzyme. It was dialyzed at 4° in Visking sausage casings (previously boiled with water for 2 hours to remove soluble materials) successively for 12 hours each against several changes of 0.1 M acetate buffer at pH 5.0, distilled water, and barbiturate buffer at pH 7.4. Both the dialysis sacs and the outside liquid were stirred during dialysis, and any precipitate formed was removed by centrifugation before further dialysis. The final enzyme solution contained an irreducible minimum of 2 γ of P per mg. of protein nitrogen. Some of the preparations contained considerably more phosphorus, which could be removed by further dialysis, but this was not attempted owing to inactivation of the enzyme by vigorous stirring.

Phosphorus Determination—Phosphorus was determined by the method of King (5). Digestion and color development were carried out in the same tube and the final volume was 3 ml. Colorimetric measurements were made with a Beckman spectrophotometer at 660 mμ. Three phosphorus standards and a blank were run with each determination.

Radioactivity measurements were made with a Tracerlab autoscaler. Decay corrections were made for all experiments lasting more than 12 hours.

Exchange Studies

The enzyme solution used in the following two experiments contained 6.4 γ of P per ml. Prolonged dialysis of an aliquot showed that it contained 2 γ of P per ml. which was non-dialyzable, while the rest could be determined as inorganic phosphate in the dialysate.

In Experiment I, 5 ml. of enzyme solution were made up to 10 ml. to contain 0.2 per cent serum albumin, 0.005 M magnesium sulfate, and 0.02 M sodium sulfite, the final pH being 7.5. To this solution 0.02 ml. of labeled substrate solution containing 22 γ of ester P was added.

In Experiment II, 4 ml. of enzyme solution and labeled substrate containing 54 γ of ester P were used, other experimental conditions being the same.

At the end of 3 hours at room temperature, one-ninth its volume of 1.0 M acetate buffer at pH 5.0 was added to each solution, which was then dialyzed against a small volume (10 to 15 ml.) of buffer with stirring. After an hour the dialysates were collected and estimations of radioactivity and inorganic and total phosphorus were carried out in duplicate on aliquots. The enzyme solutions were dialyzed with stirring against several changes of acetate buffer at pH 5.0 for 3 days at 4°.

The results are presented in Table I. The difference between the total P and inorganic P is a measure of the ester P (present as glucose-1- and glucose-6-phosphates), which contains the radioactivity. The enzyme became radioactive while the specific activity of the ester P decreased from an initial value of 2010 to 1350 and 1840 counts per minute per microgram of P respectively in Experiments I and II. This suggests an exchange between enzyme P and ester P and the following calculations show the correspondence between expected and observed values.

The non-dialyzable enzyme P and substrate-P were 10 and 22 γ respectively in Experiment I. If exchange occurs between the two and equilibrium is reached, the total radioactivity initially present in 22 γ of substrate P should then be distributed between 32 γ. Hence the specific activity of the ester should be only 22/32 of the initial value of 2010 counts per minute per microgram of P, which is equal to 1380. The observed specific activity is 1350. A similar calculation for Experiment II shows that the specific activity of ester P after reaction with enzyme should be 54/62 of the initial value, or 1750 counts per minute per microgram of P. The observed value was 1840. (In these calculations, the effect of the dialyzable inorganic phosphate of the enzyme is ignored, since no exchange of this with ester P occurs as shown by Schlamowitz and Greenberg (2).)

A further check was obtained from the radioactivity of the dialyzed enzyme. If equilibrium were established, the specific activities of enzyme P and ester P should be the same. This was experimentally confirmed.

Radioactive enzyme was prepared by a similar procedure with several different enzyme preparations. When serum albumin and sulfite were replaced by cysteine, identical results were obtained, showing that the albumin did not influence the results. The labeled enzyme could be dialyzed at 4° against acetate, barbiturate, phosphate, or glycerophosphate buffers ranging in pH from 5 to 7.5 without loss of radioactivity. In one experiment, dialysis for 15 days failed to remove its radioactivity. But when the enzyme was mixed, in the presence of magnesium sulfate and sulfite, with non-labeled glucose-1-phosphate in sufficient excess and shortly thereafter dialyzed against buffer or water, it rapidly lost its radioactivity while the dialysate containing the glucose esters was radioactive.

TABLE I

Exchange of Phosphate between Phosphoglucomutase and Labeled Glucose-1-phosphate

Fraction	Determination	Experiment I	Experiment II
Glucose-1-phosphate	Specific activity, *counts per min. per γ P*	2,010	2,010
	Total P added, γ	22	54
Enzyme	Non-dialyzable P, γ	10	8
Dialysate	Ester P, γ	9.3	16.0
	Radioactivity, *counts per min.*	12,600	29,400
	Specific activity, *counts per min. per γ P*	1,350	1,840
Dialyzed enzyme after reaction	Non-dialyzable P, γ	10	8
	Radioactivity, *counts per min.*	11,800	14,000
	Specific activity, *counts per min. per γ P*	1,180	1,750

These results can, however, also be interpreted on the assumption that the radioactive ester P was adsorbed or present in ionic combination with the enzyme after displacing an equivalent amount of inactive ester P originally combined with the enzyme. If the enzyme initially contained glucose-1- or glucose-6-phosphate in ionic combination, there would be a mere ionic exchange between this ester P and the added radioactive ester P. The previous experiments would not enable one to distinguish between such an exchange and the postulated mechanism.

An unequivocal proof would require the study of the exchange between the enzyme and substrate labeled with radioactive carbon in the glucose. Adsorption of labeled substrate would lead to the enzyme's becoming radioactive, which would not be the case if only an exchange of phosphorus between enzyme and substrate were to take place. It was, however, not possible to carry out such a study, and the following indirect evidence is offered in support of the theory proposed in the introductory paragraphs.

An enzyme solution containing 44 γ of P after prolonged dialysis was mixed with an equal volume of solution containing 0.04 M cysteine, 0.01 M magnesium sulfate, and labeled substrate equivalent to 116 γ of ester P. The pH was 7.4. After 15 minutes at room temperature it was adjusted to pH 5.0 with 1 M acetate buffer and cooled to 0°. The enzyme was precipitated with 55 per cent acetone, redissolved in acetate buffer at pH 5.0 and similarly reprecipitated with acetone twice. The radioactivity of the enzyme and the three supernatant liquids was determined.

The initial radioactivity of 116 γ of substrate P was 62,400 counts per minute; if it were to exchange with 44 γ of enzyme P, the expected radioactivity of the enzyme and glucose esters would be 17,200 and 45,200 counts per minute, respectively. The observed value for the enzyme was 18,100 counts per minute and remained unchanged after reprecipitation. The supernatant liquid after the first acetone precipitation, which contained the glucose esters, had the remaining radioactivity, while the supernatant liquid from the subsequent precipitations contained only traces of radioactivity. If the enzyme were to contain the labeled esters in ionic combination and in equilibrium with the free glucose esters, it would be difficult to explain the fact that about two-thirds of the total radioactivity associated with the esters is removed by the first precipitation while subsequent precipitations fail to remove the glucose esters.

Moreover, it was found that when the radioactive enzyme was precipitated with 10 per cent trichloroacetic acid and filtered the filtrate contained all the radioactivity. Nearly all the phosphorus present initially in the enzyme (42 γ) could be determined in the filtrate as inorganic phosphate (41 γ of P). If the enzyme contained adsorbed ester P, this could not be determined in the filtrate as inorganic phosphate, since glucose-1- and glucose-6-phosphates are only slightly hydrolyzed under the conditions of the experiment. The results can be explained on the basis that the phosphorus is enzyme-linked and recovered in the filtrate as inorganic phosphate after denaturation of the enzyme by trichloroacetic acid.

It was also observed that, though the enzyme almost completely lost its radioactivity when dialyzed after mixing with an excess of non-labeled glucose-1-phosphate at pH 7.5 in the presence of 0.005 M magnesium sulfate, it retained about 15 to 40 per cent of the initial radioactivity if it were first inactivated by treatment with 0.01 M iodine and the iodine removed with thiosulfate before reaction with substrate and subsequent dialysis. This can best be explained by the suggested mechanism, which requires unimpaired enzymic activity for an exchange of the radioactive enzyme P with the non-radioactive substrate P.

DISCUSSION

The above evidence strongly suggests, though it does not unequivocally prove, an exchange of phosphorus between the enzyme and its substrate. If confirmed, it would necessitate a revision of the current concept that an enzyme merely catalyzes a reaction while remaining unaffected by it. The present work indicates a somewhat more direct participation of the enzyme than has hitherto been demonstrated, namely an exchange of a specific atom or group with a corresponding atom or group of the substrate. The specific group of the enzyme might conceivably be bonded through a high energy bond, as indicated by the rapid hydrolysis of the phosphate of phosphoglucomutase by treatment with trichloroacetic acid in the cold.

If the mechanism herein proposed is of rather general validity and is not restricted specifically to phosphoglucomutase, it is clear that the use of enzymes bearing appropriately labeled reactive groups would be of value in the study of enzyme kinetics, the determination of turnover numbers, and the determination of molecular weights.

SUMMARY

Phosphoglucomutase contains non-dialyzable phosphorus. When the enzyme acted on glucose-1-phosphate labeled with P^{32}, the enzyme became radioactive, while the specific activity of the ester P decreased. The observed values for the subsequent radioactivity of enzyme and glucose esters correspond with those calculated on the hypothesis that an exchange between enzyme P and substrate P takes place.

The radioactivity of the enzyme could not be removed by dialysis, but after reaction with an excess of non-labeled glucose-1-phosphate the enzyme lost its radioactivity on dialysis.

After denaturation by iodine the radioactivity of the enzyme was not removed by a similar treatment.

The evidence strongly suggests an exchange between enzyme P and substrate P. The implications of such an exchange are discussed.

Addendum—After this paper was in proof there came to our attention the work of Sutherland, Posternak, and Cori (6) on the activation of phosphoglucomutase. In this work the cysteine-activated enzyme was found to be unable to convert glucose-1-phosphate to glucose-6-phosphate except in the presence of catalytic amounts of glucose-1,6-diphosphate. If the inactive enzyme, prepared by ammonium sulfate crystallization, is phosphorus-free, the active enzyme-glucose diphosphate intermediate proposed by Sutherland *et al.* would be identical in composition with that proposed by us. It would also follow that the reaction products would be glucose-6-phosphate and enzyme phosphate. Either the latter must be catalytically active in the sense of being able to combine with glucose-1-phosphate or must further dissociate to give inactive enzyme plus phosphate. If the latter is the case, another mechanism, at present unknown, must exist for the regeneration of glucose-1,6-diphosphate.

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CARTESIAN DIVER TECHNIQUE

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Micro respirometry studies, with the aid of the Cartesian diver (1, 2), have presented several problems in manipulation. One of these has been the problem of mixing two or more solutions quantitatively in the diver, after the start of an experiment. Methods have been described accomplishing this (3, 4). These methods could be mastered only by special training, patience, and skill.

A much less difficult technique is now proposed in which a new type of diver vessel is employed, having an upper reaction chamber, a lower expansion chamber, and a relatively long neck. Precise control of overpressure necessary to mix the solutions in the diver is accomplished by the use of a sphygmomanometer bulb and valve, connected into the manifold system by a 3-way stop-cock (Fig. 1). The inner surfaces of the diver need to be coated with a hydrophobic surface such as Clarite, dissolved in toluene, only once. Thereafter there seems to be enough residual coating to take care of the requirements. Sudan III is mixed with the paraffin oil to facilitate visual observation of its position in the diver neck and phenolphthalein is used in the NaOH to detect any changes in pH.

Hour-Glass Type of Diver

A method for making Cartesian divers has been described (Claff (5)), in which the various steps are carried out in a jig. The preliminary steps in the process of making the new type diver vessel are the same as for the conventional diver. The "blank" is carried through all steps, including Steps E, F, and G (see (5), Fig. 1). The capillary tubing is now as shown in Fig. 2, *A*. At this point the flame is placed in position x on the capillary, at least 4 to 6 mm. to the right of the bubble just formed. The capillary is twirled to and fro and a second bubble is formed. The end result should be as shown in Fig. 2, *B*.

To achieve this result it is necessary to start heating the capillary at least 4 to 6 mm. from the first bubble. The reason is easy to follow. The first bubble was formed from part of the glass from the solid molten mass of the tail. The second bubble has no such source of glass and the glass must come from the capillary tubing itself. If the second bubble is started too close to the first bubble, it simply coalesces with it, and one very large bubble is formed instead of two small ones.

To complete the diver, it is necessary to cut the capillary so that the neck is at least 15 mm. long (Fig. 2, *B*), calibrate it, and add glass to the tail.

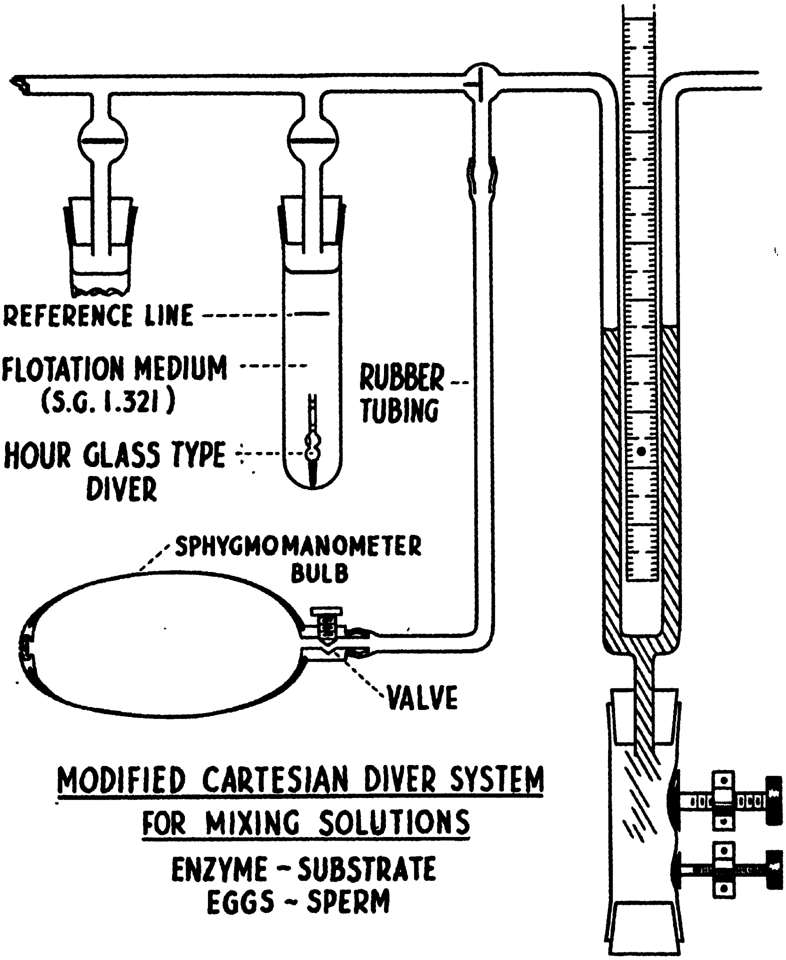

FIG. 1

Specifications of Hour-Glass Type Diver (*Fig. 2*, *C*)—The following specifications are desired for this type of diver: capillary tube, 1.22 mm. outside diameter (0.048 inch); neck length, 15 mm.; bubble diameter, 3 mm.; volume, 30 to 34 c.mm.; and total weight, approximately 97 mg. Suggested fillings are 2 c.mm. of oil, Solution 1, Solution 2, or alkali. The oil seal should be at least 2 c.mm. The other fillings are arbitrary, but must be predetermined before calibrating the diver, for which the procedure is the same as for the conventional diver (5).

Filling the Diver—The alkali drop, if used, is deposited by a calibrated pipette held in a diver filler device, as are Solution 2 and the oil seal. Solution 1 is placed on the side of the reaction chamber by the use of the "braking pipette" described by one of us (C. L. C. (6)). The pipette should have a straight sided tip at least 25 mm. long, coated with Clarite, paraffin, or lanolin or some other hydrophobic surface.

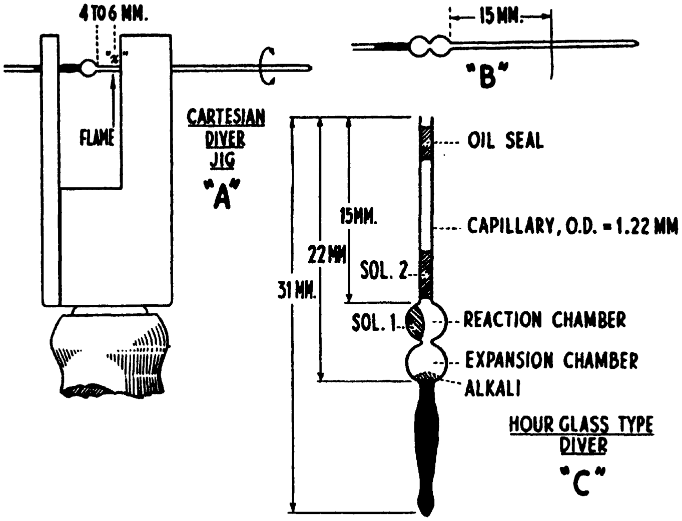

FIG. 2. Cartesian diver jig and new type of diver vessel to facilitate "mixing"

EXPERIMENTAL

To test the efficacy of this method the following experiments were performed. A suspension of *Arbacia* eggs was placed in the reaction chamber (Solution 1); a suspension of *Arbacia* sperm was placed in the lower end of the neck (Solution 2). An oil seal was placed in the upper portion of the neck, and an alkali drop at the bottom of the expansion chamber. While the diver vessel was observed through the microscope, mounted on the bath, the pressure of the manifold was slowly increased by the use of the sphygmomanometer bulb; the sperm suspension was slowly forced into the reaction chamber, where it coalesced with the egg suspension. As soon as the drops coalesced, the pressure was slowly returned to its initial pressure, by means of the valve on the sphygmomanometer bulb.

Subsequently, cleavage was observed, proving the eggs were fertilized. The accuracy of the entire system, including the calibration of the pipettes used, was tested by loading the reaction chamber with 2 c.mm. of 0.01 M

$NaHCO_3$, the lower portion of the neck with 2 c.mm. of 1.0 N H_2SO_4, and the seal with 3 c.mm. of oil.

After a short equilibration period, overpressure was applied until the solutions were mixed, and then slowly and evenly the pressure was returned to the original pressure by releasing the valve on the sphygmomanometer bulb.

The CO_2 evolution was recorded for 20 minutes. The average of six experiments showed a recorded evolution of 97.0 per cent of the theoretical yield of CO_2. One experiment with 1 c.mm. of 0.01 M $NaHCO_3$ and 2 c.mm. of 1.0 N H_2SO_4 showed a recorded evolution of CO_2 equal to 97.3 per cent of the theoretical yield (Fig. 3).

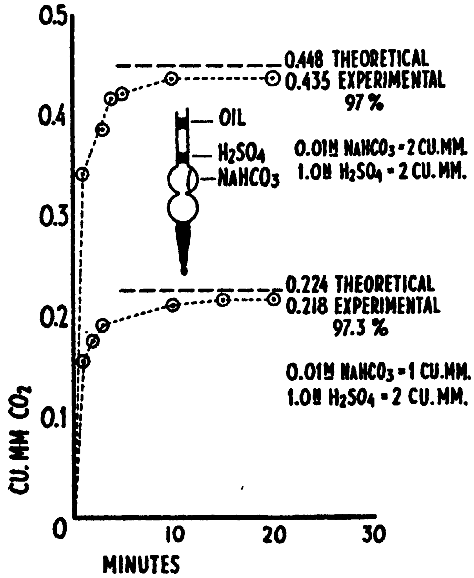

FIG. 3. CO_2 evolution from combination of $NaHCO_3$ and H_2SO_4

Since the contents of the diver may be readily examined at will by the aid of the microscope mounted on the bath, it is possible to correlate the pattern of the *observed* behavior and morphological changes of the material contained in the diver with the respiratory rate recorded.

This was shown by the following experiment with *Paramecium calkinsi*, Mating Types I and II.

The diver vessel was loaded with Mating Type I in the reaction chamber and Mating Type II directly above in the lower portion of the neck.

One diver was used as a control and the contents were not mixed. After the combined respiration of Mating Types I and II was recorded for 50 minutes in each diver, the contents of the experimental diver were mixed. A reduction of respiration rate in the experimental diver was recorded for the next half hour; then a partial return to the former rate of respiration occurred. It was possible to observe the initial clumping of the *Paramecium* within 30 seconds of the mixing. This we could correlate with the depressed respiration. After half an hour we observed conjugating pairs breaking away from the mass. This observation coincided with a recorded partial return to the original rate of respiration (Fig. 4).

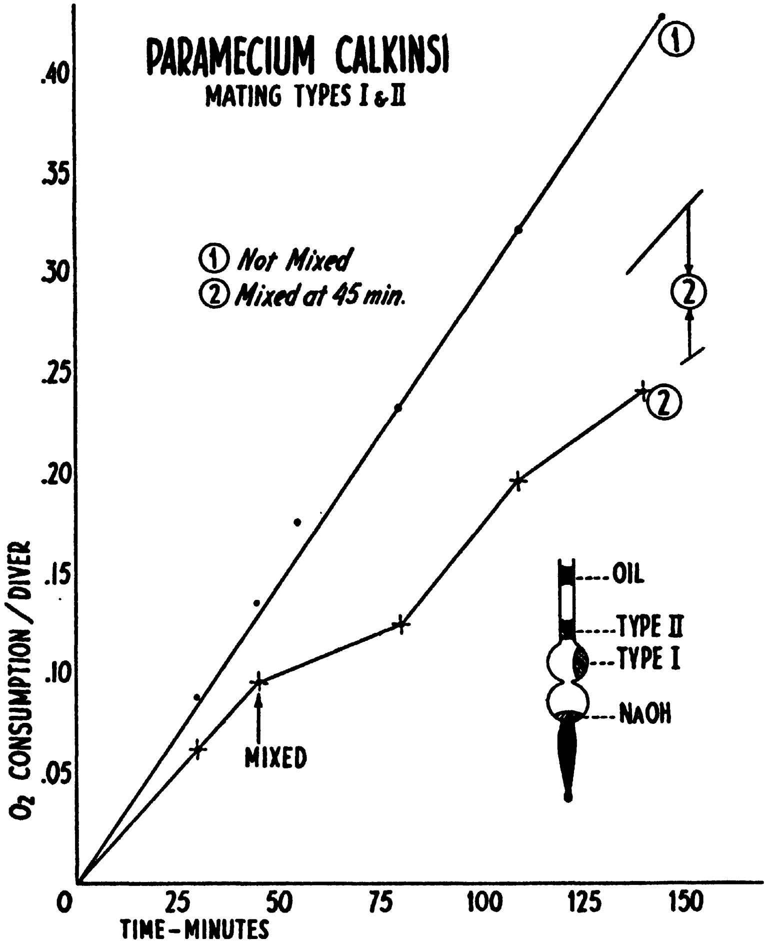

FIG. 4. Respiration rate changes due to mating reaction in *Paramecium calkinsi* (Mating Types I and II).

The effect of uranyl nitrate[1] on a group of seven *Chaos chaos* was tested as follows (Fig. 5). The *Chaos chaos* were washed several times and allowed to become acclimated in acetate buffer, pH 6.6, made up with boiled tap water.

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[1] The senior author, C. Lloyd Claff, takes full responsibility for reporting the work with uranyl nitrate and *Chaos chaos*, *i.e.* the two paragraphs describing the experiment and Fig. 5.

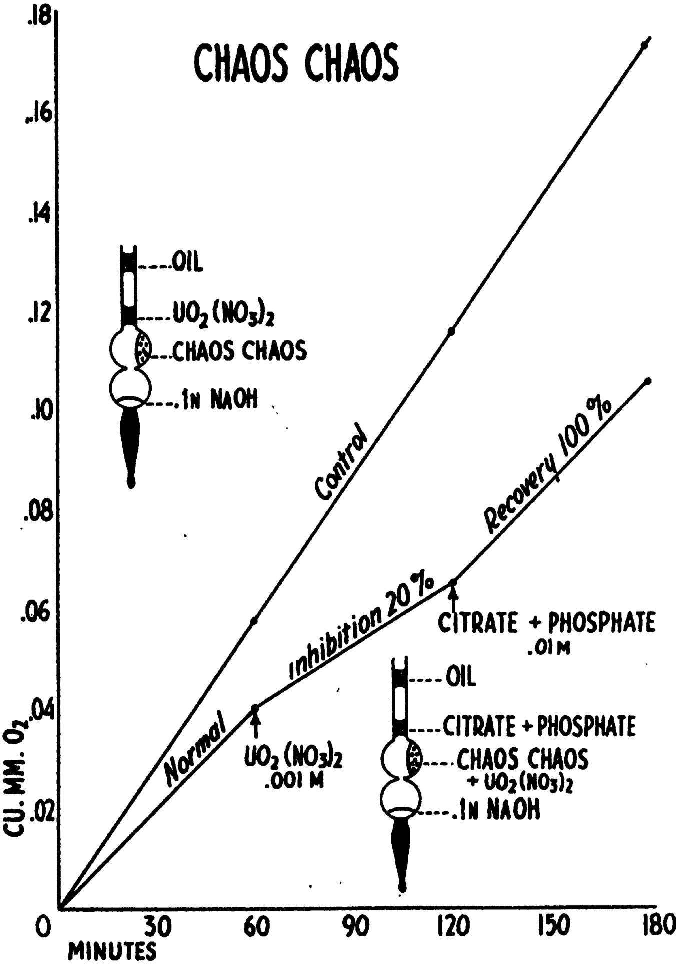

Fig. 5. Inhibition of respiration rate in *Chaos chaos* due to addition of uranyl nitrate, and recovery upon addition of citrate and phosphate.

The control diver was loaded with seven *Chaos chaos*, 0.1 N NaOH 2 c.mm. of uranyl nitrate, and an oil seal. The contents of this diver were never mixed. Its respiration rate is shown on the control graph (Fig. 5). The experimental diver was loaded in the same manner, and the respiration was recorded for 1 hour. The uranyl nitrate was then mixed with the drop containing the seven *Chaos chaos*. Respiration was inhibited 20 per cent.

After the 2nd hour the experimental diver was recovered from the bath, the oil seal removed, the neck of the diver cleaned with a spill of filter paper, and a charge of citrate and phosphate buffer (pH 6.4) was placed in the lower portion of the neck of the diver, together with a new oil seal. After a short equilibration period, the citrate-phosphate buffer was mixed with the drop containing the uranyl nitrate-treated *Chaos chaos*. Through the formation of the uranyl citrate complex, the toxic effect of the salt was eliminated, and a respiration recovery of 100 per cent was recorded.

We wish to acknowledge our indebtedness to Dr. E. S. Guzman Barron for his interest and suggestions during the course of our experiments. We also wish to thank Dr. A. A. Schaeffer for furnishing the cultures of *Chaos chaos*, and Dr. Ralph Wichterman for cultures of *Paramecium calkinsi*, Mating Types I and II.

SUMMARY

A technique is described, with a new hour-glass type of Cartesian diver vessel, which makes it relatively easy to mix quantitatively one or more solutions in the diver vessel at any time during an experiment. Some results and suggested uses of the method are described.

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ACCUMULATION OF LABILE PHOSPHATE IN STAPHYLOCOCCUS AUREUS GROWN IN THE PRESENCE OF PENICILLIN*

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(Received for publication, January 27, 1949)

The multiplication of certain strains of *Staphylococcus aureus* is prevented by the presence of less than 0.1 γ of penicillin per ml. of culture medium (1). It is known that such organisms increase in size after the addition of penicillin (2–4). We investigated this phenomenon to determine whether the increase in cell size represented an actual increase in cell substance, and more particularly to determine whether various constituents of the cell increased at comparable rates. This study indicated that the acid-soluble organic phosphate content of the cells increased at an accelerated rate under the influence of penicillin. It appears that much of this abnormal increase in soluble organic phosphate is attributable to a compound which contains acid-labile phosphate. In this paper, data are presented which indicate that this material is a new organic labile phosphorus compound.

EXPERIMENTAL

Methods

Micromethods were used for all analyses. Nitrogen was measured by the method of Johnson (5). Reducing power was estimated by the reduction of ferricyanide ions. The method, which will be published elsewhere, is similar to the method of Horvath and Knehr (6) but is 20 times more sensitive. The Fiske and Subbarow reagents were used for the determination of inorganic phosphate (7). The term labile phosphate is used in this paper to designate inorganic phosphate released when the sample, contained in 1 ml. of 1 N hydrochloric acid, is heated in a boiling water bath (99.4°) for 10 minutes. Stable phosphate represents total phosphate less inorganic phosphate and labile phosphate. The digestion procedure for nitrogen (5) was found satisfactory for total phosphate. Estimation of nucleic acid was based on the extraction procedure of Schnei-

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der (8). As may be seen in Fig. 1, the extraction removed the nucleic acids from *S. aureus* cells in 5 minutes, but additional phosphate was released by further extraction with hot trichloroacetic acid (TCA). The total nucleic acid content of the extract was then estimated from its phosphorus content (9.8 per cent P assumed), and from its light absorption (260 mμ), on the assumption that the extinction coefficient was identical with that for yeast nucleic acid.

S. aureus H was used throughout. The medium used in the growth experiments contained (per liter) 5 gm. of Difco peptone, 5 gm. of Difco yeast extract, 1 gm. of glucose, and 0.3 gm. of dipotassium hydrogen phosphate. It was adjusted to pH 7 before sterilization. Crystalline sodium benzylpenicillin was used in all experiments.

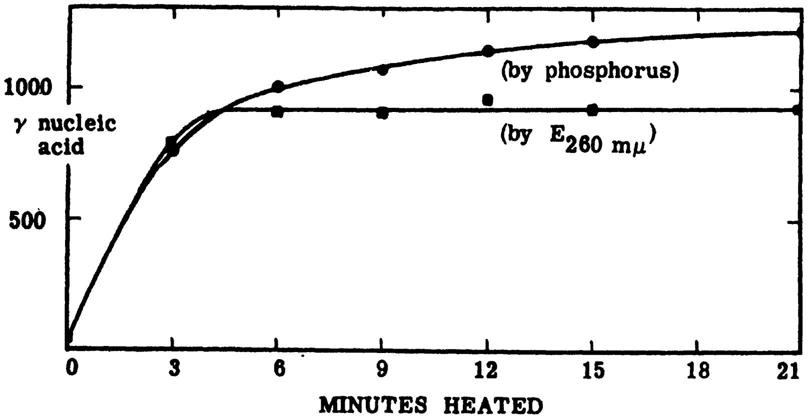

FIG. 1. Extraction of nucleic acids from *S. aureus* cells at 90° in 5 per cent TCA. Micrograms of nucleic acid = $E_{260\ m\mu}$ × 39.4 or micrograms of phosphorus × 10.8.

Results

The results of an experiment in which normal *S. aureus* cells are compared with *S. aureus* cells that were grown for a short period in the presence of penicillin are recorded in Table I. It is seen that the cultures to which penicillin had been added did not increase in cell numbers, while the normal cultures approximately doubled in population. However, as measured by dry weight, nitrogen, phosphorus, and nucleic acid, the cell substance increased almost 50 per cent in the presence of penicillin. Cell substance of the normal cultures increased about 100 per cent in the same length of time. Gale (9) has noted a depression in the rate of formation of ribose nucleic acid by *S. aureus* cells when grown in the presence of 5 units of penicillin per ml. The data from the several experiments of this type which we have performed do not suggest a marked depression in the rate of formation of nucleic acid as compared to other components of the cell. The point we wish to emphasize is that most cellular components of *S. aureus* do increase in quantity at comparable rates during growth in the presence of 0.1 unit of penicillin per ml.

TABLE I

Increase in Cell Substance of S. aureus Grown in Presence of 0.1 Unit of Penicillin per Ml.

Six replicate 500 ml. Erlenmeyer flasks, containing 100 ml. of culture, were incubated at 30° on a shaker until one-fourth maximum growth was obtained (3 to 4 hours). Two flasks were then harvested for zero time analyses, and at the same time penicillin was added to two of the remaining flasks. After 65 minutes further incubation, the four remaining flasks were analyzed.

The analytical results are recorded as micrograms in the cells from 1 ml. of culture. Total cell counts were made with a Petroff-Hausser cell counter. Micrograms of nucleic acid per ml. = $E_{260\,m\mu} \times 39.4$ or micrograms of phosphorus × 10.2.

Analysis	Composition of cells			Increase over initial	
	0 time	65 min.			
		With penicillin	Without penicillin	With penicillin	Without penicillin
				per cent	*per cent*
No. of cells $\times 10^{-8}$	8.8	8.2	17.6	2	98
	9.1	10.1	17.8		
Dry weight	330	510	690	44	102
	360	510	750		
Nitrogen	44.8	72.8	86.2	63	92
	45.4	74.0	87.0		
Phosphorus	12.5	18.4	24.5	49	94
	12.5	18.9	24.8		
Nucleic acid (phosphorus)	82	115	144	42	77
	82	116	145		
Nucleic acid ($E_{260\,m\mu}$)	88	123	145	38	64
	91	124	149		
Acid-soluble fraction					
Inorganic P	1.92	2.42	3.90	27	105
	1.90	2.44	3.92		
Labile "	0.16	0.46	0.33	200	100
	0.18	0.56	0.35		
Stable "	0.84	1.96	1.83	121	108
	0.93	1.95	1.86		

However, during this period of growth in the presence of penicillin a marked change in the distribution of the acid-soluble phosphates of *S. aureus* occurs. The data in Table I illustrate this effect. The increases in labile phosphate and stable phosphate are several times greater than one would expect from the observed increases in cell substance. Over 60 per cent of the phosphate in the acid extract of normal *S. aureus* cells is inorganic phosphate, 5 per cent or less is labile phosphate, and the remaining 30 to 35 per cent is stable phosphate. In *S. aureus* cells after growth in the presence of penicillin, a smaller percentage of the acid-soluble phosphate is found free, about 15 per cent is bound as labile phosphate, and 40 to 45 per cent as stable phosphate.

It is to be noted that this effect upon the phosphate balance of the cell was observed when only 0.1 unit of penicillin was added per ml. of culture.

Table II

Effect of Penicillin and Glucose Concentration on Acid-Soluble Labile Phosphate Content of S. aureus Cells

Penicillin concentration	Glucose concentration	Labile phosphorus in cells from 1 ml.		
		0 time*	35 min.*	65 min.*
units per ml.	*gm. per l.*	γ	γ	γ
0	1	0.24	0.31	0.43
0.1	1	0.24	0.53	0.52
1.0	1	0.23	0.80	0.84
0.1	0	0.11	0.21	0.28

All flasks contained 3 liters of medium and were incubated at 37° with aeration through carborundum dispersers.

* Minutes after addition of penicillin. At zero time the cultures were growing rapidly and had reached approximately one-third maximum growth.

From the data in Table II, it may be seen that under comparable conditions more labile phosphate is found in the cells if grown in the presence of 1 unit of penicillin per ml. instead of only 0.1 unit of penicillin per ml. The effect observed is not simply the result of an inhibition of the hydrolysis of the labile phosphates already present in the cells, since analysis immediately after the addition of penicillin did not reveal large amounts of labile phosphate. The data in Table II indicate that most of the labile phosphate is formed in the first 35 minutes after the addition of penicillin, and further that if glucose is omitted from the medium much less labile phosphate may be extracted from the cells.

All of the experiments described in this paper have been repeated, in whole or in part, several times in the past 18 months with reproducible results. However, the absolute amount of labile phosphate obtained from normal cells varies from day to day. Occasionally when only 0.1 unit of penicillin was added per ml. of culture, the phosphate balance was not appreciably altered; addition of 0.5 unit of penicillin per ml. has always been effective in causing accumulation of labile phosphate.

Fractionation of the acid-soluble phosphate compounds showed more clearly the effect of penicillin upon *S. aureus* cells. The cells were treated as indicated in Table III. It is seen that most of the labile phosphate which accumulated with penicillin present appeared in the fraction which contained the alcohol-insoluble barium salts. Further purification has been obtained by reprecipitation of the barium salts, the fraction soluble in 60 per cent alcohol and insoluble in 80 per cent (by volume) being the purest, and by passage through the cation exchange resin, Amberlite IR-100 (Resinous Products and Chemical Company, Philadelphia). The best preparation contained, in 1540 gm., 1 mole of labile phosphate, 1.06 moles of stable phosphate, and 6.1 moles of nitrogen.

TABLE III

Fractionation of Acid-Soluble Phosphates from Normal Cells and Penicillin-Treated Cells of S. aureus

The cells from 30 liters of a 3 hour-old culture of *S. aureus* (containing 13,000 μM of total P) are compared with *S. aureus* cells from 30 liters of a culture in which 13,000 units of penicillin were present for the final hour of a 3.5 hour incubation period (the penicillin-treated cells contained 13,000 μM of total P). The cultures were incubated at 37° with aeration through carborundum dispersers. Each precipitate was dissolved and reprecipitated before proceeding to the next step. About 1800 μM of inorganic phosphate were present in each extract.

Treatment	Normal cells		Penicillin-treated cells	
	Labile P	Stable P	Labile P	Stable P
	μM	μM	μM	μM
Cells extracted with TCA; after TCA removal with ether, extract neutralized, concentrated to small volume, analyzed	125	865	445	1400
Excess barium ion added, adjusted to pH 9.2, centrifuged, ppt. analyzed	40	170	75	340
Ethyl alcohol added to supernatant to 50 volumes %, centrifuged, ppt. analyzed	11	144	38	250
Ethyl alcohol added to supernatant from above to 83 volumes %, centrifuged, ppt. analyzed	40	280	297	590

Since only about 10 μM of material may be obtained from 1 liter of culture, only a small quantity has been prepared. With the material available, we have attempted to identify some of the components of the preparation, thus far with little success. The material absorbs strongly in the ultraviolet with a maximum at 262 mμ. The molecular extinction of the preparation, based on labile phosphate content, is 10,200. A color test (10) for uracil and cytosine was positive on a 10 mg. sample which had been hydrolyzed with 25 per cent sulfuric acid for 3 hours at 175°. Evidence obtained by the paper chromatographic method of Hotchkiss (11) indicates that well over half of the light absorption is caused by uracil. Vischer and Chargaff (12) have demonstrated that cytosine is gradually converted to uracil during acid hydrolysis. However, if considerable cytosine had been present in the material before hydrolysis, an absorption maximum of 265 to 270 mμ would have been expected. As tested by the method of Schmidt and Levene (13), only a small percentage of the total absorption at 260 mμ may be attributed to purines.

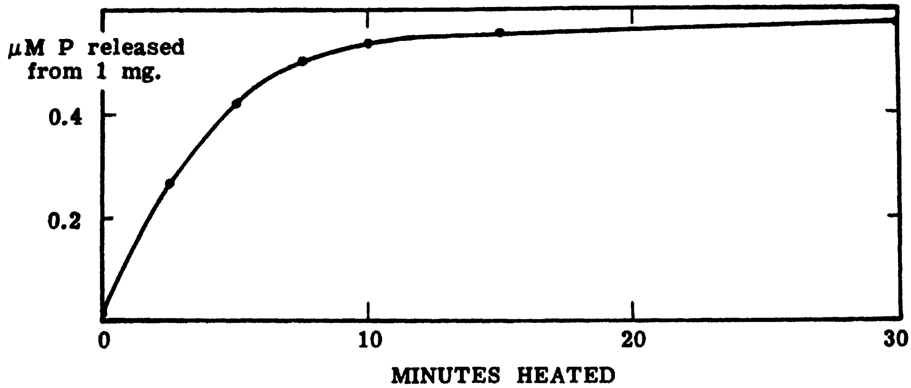

FIG. 2. Release of inorganic phosphate from the preparation during hydrolysis in 1 N hydrochloric acid in a boiling water bath.

Quantitative determination of pentose by an orcinol method (14) indicates that 0.2 mole of pentose is present per mole of labile phosphate. However, the amount of apparent pentose increases if the heating time is lengthened. Thus it is not certain whether the pentose is a contaminant or whether it is firmly bound and hence does not react quantitatively under the conditions of the orcinol test used. There is present in the preparation a potential reducing group which is completely freed in 3 minutes at 100° in 0.1 N hydrochloric acid. Under these conditions a small amount of phosphate is released. Hence it is possible to reprecipitate most of the material as the barium salt with alcohol, but the reducing material remains in solution. The reducing material thus obtained gives a yellow color (maximum absorption at 400 mμ) in the carbazole test (15). The absorption spectrum does not correspond with that for any of the common hexoses or pentoses. The potential reducing power of the preparation per mole of labile phosphate is equivalent to about 0.7 mole of glucose as determined by the ferricyanide reduction method.

There is no direct evidence that the reducing material, the constituent responsible for the absorption of ultraviolet light, and the labile phosphate are all parts of a single compound. However, the compound or compounds which possess these properties diffuse through a Northrop-Anson (16) porous disk at the same rate.

The barium salt of the organic phosphate preparation is very soluble; one can readily dissolve 0.4 gm. in 1 ml. of water.

The rate of release of phosphate from the preparation in 1 N hydrochloric acid in a boiling water bath is shown in Fig. 2. It can be seen that the labile phosphate is released in about 12 minutes, but that a true measure of the amount of labile phosphate present is obtained by 10 minutes hydrolysis, since a small amount of stable phosphate is liberated. Under these conditions, 9 minutes hydrolysis is necessary to obtain a true measure of the labile phosphate content of adenosine triphosphate. Thus the hydrolysis of phosphate from these compounds proceeds at almost the same rate.

DISCUSSION

Since penicillin is effective in such small quantities, it seems essential that its action be highly specific. The fact that sensitive bacteria do continue to grow for some time in the presence of small amounts of penicillin offers a special opportunity to look for the specific reaction with which penicillin is concerned. The labile phosphate compound described above may be related to this hypothetical reaction and hence accumulate rapidly when the reaction is slowed. This compound appears to contain the major portion of the labile phosphate in such cells and may also be present in appreciable quantity in normal cells.

It is believed that the properties ascribed to this material distinguish it from all known organic compounds containing labile phosphate. The absence of certain compounds lends support to this view even though the preparations studied have not been pure. Thus the absence of adenine excludes adenosine diphosphate and triphosphopyridine nucleotide. Of course, most of the known compounds of this type can be excluded on the basis of solubility alone. Glucose-1-phosphate and similar compounds are not present, since acid hydrolysis liberates the reducing group long before appreciable phosphate is released. Thiamine pyrophosphate does not have the absorption spectrum which is characteristic of the preparation. Thus it is concluded that this material represents a new form of organically bound labile phosphate.

Further investigation of the properties of this material is in progress.

SUMMARY

Abnormal amounts of acid-soluble labile phosphate accumulate in *Staphylococcus aureus* cells when grown in the presence of penicillin. A

partially purified preparation has been obtained from such cells which contains most of this labile phosphate. Evidence is presented which indicates that this material is a new labile phosphorus compound.

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ISOLATION OF SOME CRYSTALLINE YELLOW PEPTIDES FROM ENZYMIC DIGESTS OF DINITROPHENYL INSULIN AND DINITROPHENYL TRYPSINOGEN

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Previous work has shown that the peptide-like growth factor strepogenin probably occurs in certain proteins such as insulin and trypsinogen at the amino end of the protein (1), and that it is liberated by suitable enzymic digestion. This conclusion suggested a means for attacking the almost insurmountable task involved in separating the one compound strepogenin from the mixture of numerous peptides and other cleavage products which result from the enzymic digestion of a protein. If the protein were converted to the dinitrophenyl (DNP) derivative by reaction of its amino groups with 2,4-dinitrofluorobenzene (2), the strepogenin of which the amino group is exposed should be converted into DNP strepogenin, which might then be liberated during enzymic digestion. A relatively small number of yellow, DNP compounds should result from such a digestion, because the number of amino groups of the proteins is small. Furthermore, the DNP derivatives are no longer amphoteric substances, but are organic acids which can be extracted into organic solvents and thus separated from the more numerous products of protein cleavage. This latter property very materially increases the scope of means available for successful separation of mixtures. For these reasons the isolation of pure, crystalline yellow cleavage products of DNP insulin and DNP trypsinogen has been attempted. Even though the biological inactivity of DNP strepogenin rendered impossible the direct determination of which, if any, of the isolated materials might be the derivative of the growth factor, the value of the study to the problem of the exact chemical structure of proteins seemed sufficient reason for proceeding.

Since much evidence points to the conclusion that strepogenin is a derivative of glutamic acid (3), the presence of this amino acid in one of the cleavage products of DNP insulin might indicate a relationship to strepogenin. However, all yellow peptides isolated contained this amino acid, and so no deduction could be made except that glutamic acid is very near the amino end of some of the peptide chains in this protein.

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* With the technical assistance of R. A. Brown, J. Cowperthwaite, A. L. Holloway, and N. B. Smith.

When DNP insulin was digested with pancreatin and the digest acidified, three major fractions of colored material could be separated readily. One was obtained by extraction with ethyl acetate (Fraction I), a second by subsequent extraction of the residual aqueous phase with butanol (Fraction II), and a third remained suspended in the watery layer as an insoluble precipitate (Fraction III). Since Fraction III did not occur when the strepogenin-containing portion of oxidized insulin (1, 4) was used in place of the native protein, this fraction was not separated further, even though it amounted to more than half of the total DNP insulin. It was not merely undigested DNP insulin, because repeated treatment with fresh portions of enzyme did not destroy it, and yielded only small amounts of soluble components.

The ethyl acetate extract and the butanol extract were each separated into a number of components by a series of counter-current distributions between pairs of immiscible solvents. This method of separating pure compounds from mixtures of closely related substances has been developed by Craig *et al.* (5). For this purpose, no solvent system was found which was suitable for the separation of the free acids. Many which were tried, especially those containing chloroform, were found to cause extensive decomposition of the DNP peptides.[1] However, the partition of the ammonium salts between butanol and dilute aqueous ammonia proved quite useful and, in the case of several of the components, led to the isolation of crystalline, and apparently homogeneous compounds. Both the ethyl acetate extract and the butanol extract when separated in this fashion showed a colored fraction which did not move with the advancing front of butanol, and this was called the ethyl acetate left end-piece, or the butanol left end-piece, since it remained in the left-hand tubes of a series used in the counter-current distribution. A second set of colored tubes, found in the exact center of a counter-current series, was obtained from the butanol extract (or Fraction II). This was called the butanol mid-piece. In the ethyl acetate extract (Fraction I), a mid-piece, slightly to the right of the middle tube in a series, was found, and it was called the ethyl acetate mid-piece. At the right-hand end of a series of counter-current tubes of a partition of either the ethyl acetate extract (Fraction I) or of the butanol extract (Fraction II), there were some yellow fractions, representing substances which could not be extracted from butanol solution into aqueous ammonia. These were designated the ethyl acetate and butanol right end-pieces. In addition, there was a colored substance which was present in the butanol extract or Fraction II which moved only slowly down a series of counter-current tubes containing butanol and dilute aqueous ammonia, and this was called the slow moving butanol left end-piece. A flow sheet to clarify the preparation of these major fractions is shown.

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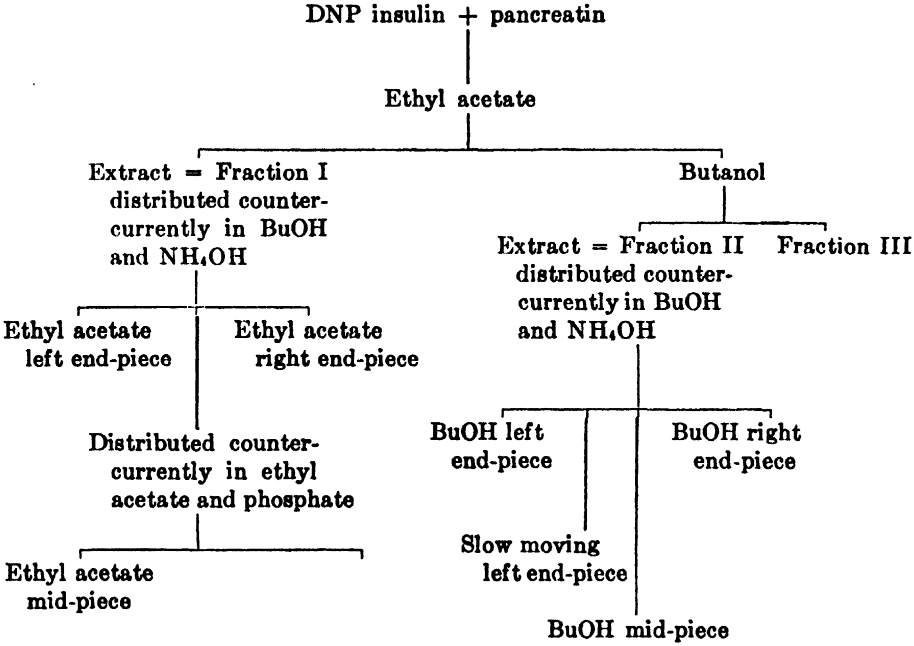
[1] This rather unexpected behavior can best be illustrated by the following experiment. Fraction II (the butanol-extractable fraction) was distributed counter-currently between aqueous 5 per cent acetic acid and an organic layer made from 90 per cent chloroform and 10 per cent butanol. Part of the color remained completely in the aqueous phase and this fraction was partitioned counter-currently in a new solvent system of 5 per cent aqueous acetic acid and 1:1 chloroform-butanol. Most of the color remained completely in the organic layer. When this fraction was again distributed in the first solvent system, it was found to be so altered that all the color remained in the chloroform phase of the first tube, whereas before solution in the 1:1 chloroform-butanol it had remained in the aqueous phase. Indeed the color was now extractable into pure chloroform. This experience, together with many others of a similar character, indicated that exposure to acid, chloroform, and butanol appreciably altered the yellow compounds.


DNP insulin + pancreatin
Ethyl acetate
Extract = Fraction I distributed counter-currently in BuOH and NH$_4$OH
Butanol
Extract = Fraction II distributed counter-currently in BuOH and NH$_4$OH
Fraction III
Ethyl acetate left end-piece
Ethyl acetate right end-piece
Distributed counter-currently in ethyl acetate and phosphate
Ethyl acetate mid-piece
BuOH left end-piece
BuOH right end-piece
Slow moving left end-piece
BuOH mid-piece

Some of these fractions obtained by counter-current distribution appeared either to be pure compounds or ones which could be rendered pure by simple additional distributions. This was true for the butanol mid-piece, the ethyl acetate mid-piece, and the slow moving butanol left end-piece. The criteria of purity were the following: (*a*) Each compound moved as a single symmetrical peak in counter-current partition of the ammonium salts between butanol and dilute aqueous ammonia. For each substance the symmetry of the peak was demonstrated by examination of tubes on the left-hand side and on the right-hand side of the one with maximal color. When the contents of these tubes were distributed counter-currently in the same solvent system, they showed a maximal color in the identical position to that assumed by the peak in the previous counter-current series. If readily separable impurities had been present, the position of the peak in the secondary counter-current distributions would not have been the same as in the primary one. (*b*) Evaporation of the ammoniacal solution of each of the isolated fractions left crystalline yellow solids with rather characteristic and reproducible melting points. (*c*) Counter-current distribution of the isolated fractions in new solvent systems failed to indicate the separation of a colored impurity. Although two compounds might have the same rate of migration in one pair of solvents, the likelihood that they would also move at the same speed in a second pair of solvents is more remote. (*d*) Because one might argue that the number of transfers in the distributions was not large enough to insure adequate separation, each isolated fraction was placed on a paper strip chromatogram (6) and examined in two different solvent systems. One of these was phenol saturated with dilute aqueous ammonia and the other was butanol saturated with dilute aqueous ammonia. There is evidence from many prior investigations that the resolving power of paper strip chromatograms is quite good. In the solvent systems employed the isolated fractions behaved as single substances. (*e*) Each isolated fraction was hydrolyzed and the hydrolysates were analyzed quantitatively for various amino acids by microbiological procedures. These tests showed that the molecular ratios between the amino acids in each substance were approximately 1:1. In the slow moving butanol left end-piece approximately 3 moles of leucine were present for each mole of the other amino acids. If the isolated fractions were mixtures, one would not expect such stoichiometric proportions unless the components of the mixture were present in equimolecular amounts. The probable error in microbiological assays is large, but despite this, gross impurities should have been detected by the procedure outlined above.

These criteria of purity may not be sufficient for substances such as peptides, but they are possibly as good as any thus far applied to such materials. Taken together they indicate that the isolated substances were of sufficient homogeneity to warrant further study.

A few of the fractions obtained by counter-current distribution were impure. This was true of the ethyl acetate right end-piece which formed a diffuse band of yellow color spread over several of the tubes at the right-hand end of the counter-current series. In these cases, some separation was achieved by differential solvent extraction and by extended counter-current distribution in new solvent systems, but the obtaining of homogeneous material from the end-pieces was not achieved.

The right-hand end-pieces, especially the butanol right end-piece, contained some artifacts. These fractions, it will be recalled, represented those substances which were completely held in butanol in preference to aqueous ammonia. They must, therefore, have been ammonium salts with a far greater affinity for butanol than for water, or else they were neutral compounds with no carboxyl groups. Now, since the peptides in the butanol right end-piece contained serine and threonine, the hydroxyls of these amino acid residues in the peptide might be so situated as to allow lactone formation with the carboxyl groups. In this way neutral compounds could result. Evidence for this hypothesis was found in that the dry substances in the butanol right end-piece would not dissolve in 0.1 N NaOH. However, when heated in this solvent for a time, solution occurred. When the resulting solution was acidified, the free acid was formed, and this, when distributed counter-currently between butanol and dilute aqueous ammonia, showed clearly two colored compounds. One of these moved in counter-current series as did the butanol mid-piece, and the other as did the butanol right end-piece. The amino acid composition of the fractions was identical, as determined by the paper strip method of Consden, Gordon, and Martin (6), and was not altered from that of the original butanol right end-piece. During the acidification following the opening of the lactone ring by gently heating with alkali, some of the free acid formed reverted to the lactone, which once again appeared as a neutral compound at the right-hand end of a counter-current series. Furthermore, if the butanol mid-piece, isolated in the normal course of fractionation of the DNP insulin digest, was dissolved in water, acidified, and extracted with butanol, a counter-current partition of the extract so obtained showed not only a mid-piece as expected, but also a right end-piece, which probably arose by lactonization while the substance was in acid solution. The qualitative amino acid composition was unchanged during these operations. In view of these facts it would seem probable that at least some of the butanol right end-piece was an artifact which arose from the butanol mid-piece by lactonization while the latter was exposed to acid. This instability of the free acids, as well as other less clearly defined decompositions and rearrangements of the fractions under investigation, made it imperative to employ the mildest conditions possible for their preparation.

The amino acids obtained by hydrolysis of each pure DNP peptide were determined qualitatively by paper strip chromatography, and the existence of most of the amino acids so indicated in the hydrolysates was confirmed microbiologically (7). The ethyl acetate left end-piece, after suitable purification, contained a substance which, when hydrolyzed, yielded only glutamic acid and the chromophoric group. The ethyl acetate mid-piece gave rise to the chromophoric group, glutamic acid, serine, threonine, alanine, valine, leucine, and isoleucine. The butanol mid-piece yielded these same constituents and, in addition, aspartic acid. The slow moving butanol left end-piece gave the same amino acids (except alanine) as the butanol mid-piece, and the butanol left end-piece showed all except threonine, of the constituents of the butanol mid-piece plus cystine. The existence of serine and alanine in these substances was demonstrated solely by paper strip chromatography. While the serine could be detected with considerable certainty, alanine was not always easy to differentiate from the threonine, and for this reason there was some doubt of its existence in the compounds.[2] The demonstration of the coexistence of leucine and isoleucine depended entirely on microbiological assay, since these two amino acids were not separable by chromatography on paper strips. Because the detection of the amino acids depended on the paper strip method, some of them may have escaped notice and thus the entire qualitative composition of the yellow peptides was not regarded as established with certainty.

The chromophoric group in each of the fractions was tentatively concluded to be a DNP-glycine residue, but the evidence for this was unsatisfactory. It consisted of the isolation of DNP-glycine from acid hydrolysates, but the difficulty was that the yield was very low, and most of the colored hydrolysis product was obtained as an unidentified mixture which could be separated in a series of counter-current distributions between butanol and dilute aqueous ammonia as a left end-piece, which did not migrate with the butanol, and a right end-piece, which did not remain at all in the aqueous phase. This latter component was a neutral material.

The failure to isolate good yields of DNP-glycine was not due to inadequacies of the methods, because the counter-current distribution procedure was found to be an elegant way to separate DNP-glycine and DNP-phenylalanine, both of which Sanger has shown to occur in acid hydrolysates of DNP insulin (2). However, in contrast to Sanger's findings with a chromatographic method of separating DNP amino acids from hydrolysates of DNP insulin, the counter-current procedure when applied to such hydrolysates showed a third colored substance, corresponding in position to the right end-piece yielded by hydrolysates of the peptides isolated in this work. When pure DNP-glycine was subjected to the action of hot 20 per cent HCl, such as is used in the hydrolysis of the peptides, a minor portion of it was converted into a colored substance which stayed at the right-hand end of a counter-current series, but the extent of this decomposition was small compared to that found with the DNP peptides isolated in this work. Studies with some synthetic peptides containing DNP-glycine residues revealed one which yielded no DNP-glycine when hydrolyzed with hot 20 per cent HCl, but which gave instead two colored components as did the compounds from insulin. Thus, bis-DNP-diglycyl-α-aminoalanine, although containing a high amount of DNP-glycine residues, gave no DNP-glycine when boiled with strong HCl. This synthetic substance was converted completely into degradation products similar to those from the isolated peptides. This fact tended to show that DNP-glycine can exist in a peptide in such a linkage as to preclude its liberation by the ordinary acid hydrolysis; but it does not imply that a structure identical with the synthetic model compound existed in the peptides from insulin. The small yield of DNP-glycine which was isolated from hydrolysates of the insulin fractions may be taken as evidence that the chromophore in these substances was DNP-glycine; or it may be taken as indication that the peptides were contaminated with small amounts of impurities which gave rise to to DNP-glycine.

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[2] For this reason the composition of two of these peptides which were described in a preliminary note (11) was slightly in error. The use of ammonia in the paper strip apparatus permitted a better means of distinguishing between threonine and alanine than did neutral aqueous phases.

Insulin may be cleaved, as Sanger has shown (4), by oxidation with performic acid in formic acid solution, and when this is done, two major fractions are obtained. One fragment is soluble in water at pH 6, and the other is not. The cleavage appears to depend on rupture of the molecule by oxidation of —S—S— bridges of cystine residues rather than to hydrolysis of peptide bonds. Sanger has reported that the fraction soluble at pH 6 owes its free amino groups to glycine, and that the basic amino acids seem to be absent from it. Since Woolley (1) has shown that the strepogenin activity of insulin resided in this fraction of the oxidized protein, the DNP derivative of it was prepared, digested with pancreatin, and the digest was fractionated by the methods described for DNP insulin. Two major differences were noted in the yellow peptides from oxidized insulin fraction and those from DNP insulin: (1) practically no Fraction III (the yellow residue not extractable by ethyl acetate or by butanol) appeared, and (2) the butanol left end-piece gave a compound containing cysteic acid instead of cystine.

Pancreatin digests of DNP trypsinogen were fractionated by the same methods as were used for DNP insulin, and two yellow peptides indistinguishable from those from DNP insulin were isolated. These were the butanol mid-piece and the ethyl acetate mid-piece. The probable identity of the fragments from insulin with those from trypsinogen was indicated by the following evidence: (1) The melting points were the same. (2) The positions in series of counter-current distributions were the same. This was true not only in butanol and dilute aqueous ammonia but also in other solvent systems. (3) The compounds from DNP insulin moved at the same rate as those from DNP trypsinogen on paper strip chromatograms when tested side by side on the same paper. (4) The qualitative amino acid composition as revealed by paper strip chromatography of hydrolysates was identical for the corresponding fractions of the two proteins. Thus it would seem that two moderately large pieces of both proteins were identical. Whether the same fragments could be found in other proteins not originating in the pancreas is a question of importance to investigate.

A third yellow peptide was isolated from digests of DNP trypsinogen. This one yielded DNP-methionine, serine, and isoleucine when it was hydrolyzed with acid. No evidence was found of a similar substance in digests of DNP insulin.

Because the yellow peptides isolated from DNP insulin seemed to form a regular series of increasing amino acid complexity, the hypothesis might arise that they were all derived by graded hydrolysis from a single portion of the protein molecule. The order of occurrence of the amino acid residues, counting from the free amino group of the protein, might thus be deduced, at least in part. This hypothesis has been examined in the following ways and found not to fit the observed facts. The more complex peptides were digested further with fresh quantities of pancreatin, and the digests were examined for the presence of the less complex substances which one would expect to arise by stepwise degradation of the larger molecules. The slow moving butanol left end-piece was found not to give smaller yellow fragments when digested further. The butanol mid-piece, on the other hand, did yield small amounts of what appeared to be the ethyl acetate left end-piece and the ethyl acetate mid-piece; therefore, the existence of two differing large peptides was indicated among the compounds isolated.

A second line of evidence pointed to the same conclusion. Partial hydrolysis with cold, concentrated HCl gave a yellow cleavage product from the slow moving left end-piece and analysis showed that this fragment contained the chromophore, leucine, isoleucine, and glutamic acid. Similar treatment of the other large peptides (*e.g.* the butanol mid-piece) gave a new fragment containing only the chromophore and glutamic acid. Thus, again, the existence of two large peptides was indicated with differing arrangements of amino acid residues.

It would seem that at least two distinct peptide chains in insulin gave rise to the products isolated in this study. Much concerning the order of amino acids in this protein may be learned by further investigation of these materials.

EXPERIMENTAL

Pancreatin Digestion of DNP Insulin and Preliminary Separation of DNP Cleavage Products with Solvents—A typical run will be described in this and subsequent sections to illustrate the procedures employed. 1.1 gm. of DNP insulin prepared according to Sanger's directions (2) from recrystallized insulin[3] were suspended in 600 cc. of water containing 2.5 gm. of K_2HPO_4, and the suspension was heated. When it was almost boiling, the protein suddenly dissolved.[4] The cooled solution was treated with 100 mg. of pancreatin, covered with a thin layer of toluene, and held at 37° overnight. Enough HCl was added to reduce the pH to 3, and the resulting suspension was extracted five times[5] with 500 cc. portions of ethyl acetate. The extracts were evaporated under reduced pressure to dryness at a temperature below 40°. This was Fraction I. The aqueous suspension remaining after the ethyl acetate extraction was extracted four times with 400 cc. portions of butanol, and the extracts were mixed with 1 liter of water and 10 cc. of concentrated ammonium hydroxide and concentrated under reduced pressure to dryness. Care was taken that the water was the last solvent to evaporate, because anhydrous butanol was deleterious. This was Fraction II. The residual aqueous suspension after the butanol extraction was filtered, and the precipitate was washed well with water and dried to yield 700 mg. This was Fraction III. The aqueous filtrate was colorless.

Isolations of Pure DNP Peptides by Counter-Current Distribution of Fractions I and II—For these separations, the general method of Craig *et al.* (5) was followed. The labor-saving machine developed by Craig was not used because it was necessary to centrifuge each tube after each transfer in order to cause the solvents to clear. The distributions were performed in centrifuge tubes, and the top layer was moved from tube to tube down the series with a fine tipped pipette.

Fraction I (the ethyl acetate extract) was dissolved in 15 cc. of water containing 5 per cent of its volume of concentrated ammonium hydroxide, and the solution was evaporated under reduced pressure to dryness and counter-currently distributed between butanol and dilute aqueous ammonia (95 cc. of water plus 5 cc. of concentrated ammonium hydroxide) through a total of twenty tubes. 15 cc. of each solvent were used in each tube. The color in each tube was judged visually, and maxima, or peaks, were found in Tubes 1 (left end-piece), 12, 13, and 14, and a broad one in Tubes 16, 17, 18, 19, and 20.

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[3] Crystalline insulin used in this work was very kindly supplied by Eli Lilly and Company, by Hoffmann-La Roche, Inc., and by E. R. Squibb and Sons. Most of the experiments were performed with a Lilly sample, derived from beef, which had been recrystallized several times.

[4] Because the DNP insulin would only dissolve in rather hot alkaline solution, some change in the protein caused by these conditions seems probable.

[5] The extraction was continued until the final extract was colorless. The ethyl acetate mid-piece was not readily extractable into ethyl acetate.

Ethyl Acetate Left End-Piece—The contents of Tube 1 were concentrated under reduced pressure to dryness, dissolved in 15 cc. of water, and the solution was adjusted to pH 3 with HCl and extracted twice with ethyl acetate. The extracted material was dried under reduced pressure, then triturated with 10 cc. of ethyl acetate, and the insoluble matter was discarded. The soluble portion when evaporated left 6 mg. of a rather hygroscopic, crystalline residue. This was the ethyl acetate left end-piece.

Ethyl Acetate Mid-Piece—To purify the ethyl acetate mid-piece, the contents of Tubes 10 to 14, containing the middle peak in the counter-current series, were combined, concentrated under reduced pressure to dryness, and the residue was counter-currently distributed between ethyl acetate and a buffer made from 1 volume of 0.1 M Na_2HPO_4 and 2 volumes of 0.1 M NaH_2PO_4. 15 cc. of each solvent were used in each tube and the fraction was distributed through ten tubes. A small amount of impurity, identified as 2,4-dinitrophenol,[6] m. p. 116°, was thus removed as a peak in Tubes 4, 5, and 6, and the pure ethyl acetate mid-piece was recovered from Tubes 1, 2, and 3 (peak in Tube 2) by acidification to pH 3, extraction with ethyl acetate, and counter-current distribution between butanol and dilute aqueous ammonia. This last was done in order to obtain the ammonium salt, because of the hygroscopic and unstable nature of the free acid. By evaporation of the contents of the tubes showing color in this distribution, a crystalline preparation weighing 10 mg. was secured. These crystals lost birefringence and appeared to melt at 163°.[7] After this change a solid residue was left which did not liquefy below 230°.

In an attempt to purify the substance further, it was distributed counter-currently between butanol and 5 per cent aqueous ammonium hydroxide through twelve tubes with 30 cc. of the aqueous phase and 10 cc. of the butanol in each tube. A symmetrical peak of color appeared in Tube 7. In order to test the symmetry of this peak the contents of Tube 5 were distributed counter-currently in the same solvent system. A single peak of color occupying the same position in the series as that in the primary distribution was observed. Similarly, when the contents of Tube 9 of the primary distribution were distributed counter-currently, a single peak of color in the same position as that of the primary series was found.

0.02 cc. of a 0.1 per cent solution of the colored substance in 3 per cent aqueous ammonium hydroxide was placed on a strip of Whatman No. 1

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[6] If the acidified pancreatin digest was extracted with ether before ethyl acetate was used, the dinitrophenol was effectively removed. When this modification was used, the definition of the position of the mid-piece in the distribution was much clearer and the use of the phosphate-ethyl acetate counter-current distribution step was unnecessary.

[7] All melting points reported in this paper were determined on a hot stage microscope.

filter paper and the strip was treated as a paper chromatogram according to the general procedure described by Consden *et al.* (6). When the solvent in the boat was phenol saturated with 3 per cent aqueous ammonium hydroxide, the yellow color of the compound was observed to move as a single spot with an R_F of 0.68. When the solvent was butanol saturated with 3 per cent aqueous ammonium hydroxide, the color moved as a single spot with an R_F of 0.63.

Separation of Fraction II; Butanol Left End-Piece—Fraction II, the butanol extract of the digest, was separated by counter-current distribution between butanol and dilute aqueous ammonia through a series of fourteen tubes with 15 cc. of each phase in each tube. Maximal color was in Tube 2, in Tube 7, and in Tubes 13 and 14. In order to separate further the components at the left-hand end, the contents of Tubes 1 to 4 were freed of solvents under reduced pressure and distributed counter-currently between butanol and dilute ammonia through twelve tubes, with 30 cc. of butanol and 10 cc. of aqueous phase in each tube. In this way the slow moving butanol left end-piece was concentrated in Tubes 3 and 4 and was separated from the butanol left end-piece which remained in Tube 1. This latter was precipitated as the free acid by evaporation of the solvents, solution in water, and addition of HCl. The only evidence for its purity was that preparations made from successive batches of digest showed the same decomposition point of 220°, and that on a paper strip chromatogram with butanol-ammonia the color did not move, while with phenol-ammonia a single spot of R_F 0.58 appeared.

Slow Moving Butanol Left End-Piece—The slow moving butanol left end-piece was obtained as 30 mg. of rod-shaped crystals, melting at 220° after darkening from 196°, by evaporation of the solvents under reduced pressure. Attempts were made to purify it further by counter-current distribution between butanol and phosphate buffer, but no heterogeneity was found. Similarly, it was purified by counter-current distribution between butanol and 5 per cent aqueous solution of butylamine. In this system, the butylamine salt of the peptide migrated faster than the ammonium salt had done in the runs above. With equal volumes of the two phases, a homogeneous peak was found in Tube 3 of a series of ten tubes. The butylamine salt was oily when dried. The homogeneity of the colored material in the counter-current distribution series with butanol and aqueous ammonium hydroxide was established in the same way as was described for the ethyl acetate mid-piece. When the compound was tested on paper chromatograms, it showed a single spot of yellow color with an R_F of 0.12 in butanol-ammonia and a single spot of R_F 1.0 in phenol-ammonia.

Butanol Mid-Piece—The butanol mid-piece was freed of a trace of impurity by counter-current distribution between butanol and 0.1 M sodium phosphate buffer made from 4 volumes of Na_2HPO_4 and 1 volume of NaH_2PO_4 in a series of eight tubes. The impurity was found in Tubes 3 to 5, but it was so small in amount (2 mg.) that its presence did not materially affect the melting point of the main fraction. This latter was recovered from Tubes 7 and 8 by acidification to pH 4, extraction with butanol, and counter-current distribution between butanol and dilute ammonia. This latter step was necessary because in the acidification some of the substance was changed to that occurring in the butanol right end-piece (lactone of the mid-piece?). The mid-piece, obtained by evaporation of the ammoniacal solution, was a crystalline substance (26 mg.). When heated, it changed rather sharply at 135°, but when heating was continued, a second change occurred at 160° and the compound finally melted at 205–210°.

The symmetry of the peak of color occurring in the counter-current distribution series with butanol and dilute aqueous ammonia was demonstrated in a manner similar to that described for the ethyl acetate mid-piece. When the compound was tested on paper strip chromatograms, it was found to move as a single spot with an R_F of 0.50 in butanol-ammonia and with an R_F of 0.86 in phenol-ammonia. The presence of the impurity which was removed by the distribution in butanol and phosphate buffer could be demonstrated readily in crude preparations on a paper strip chromatogram with phenol-ammonia, for this impurity had an R_F of 0.68 in this solvent. On a butanol-ammonia chromatogram the impurity could not be distinguished from the butanol mid-piece.

Isolation of DNP Peptides from pH 6 Soluble Portion of Oxidized Insulin—Since details of the procedure used by Sanger (4) for the oxidation of insulin and the separation of the fragments are not yet available, the method used in this work will be described. 500 mg. of crystalline insulin[8] were dissolved in 30 cc. of commercial formic acid, and 3 cc. of a 30 per cent solution of hydrogen peroxide were added. After 15 minutes, the formic acid was removed as completely as possible in a good vacuum at 40°, the glassy residue was rubbed with 35 cc. of 0.2 N H_2SO_4, and the suspension was freed of H_2O_2 by alternate additions of small portions of KI and of $Na_2S_2O_3$. The mixture was adjusted to pH 6, and after 2 days in the cold it was filtered and the precipitate was washed with 2 cc. portions of water. The filtrate was concentrated under reduced pressure to about 30 cc. and 600 mg. of $NaHCO_3$ were added. Reaction with 2,4-dinitrofluorobenzene (0.75 cc.) was conducted according to the general procedure of Sanger. After extraction of the excess reagent with ethyl acetate at pH 6, the DNP derivative was precipitated with HCl, washed, and dried.

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[8] Squibb insulin was used.

Pancreatin digestion of the product and separation of the yellow peptides were carried out as in the case of DNP insulin. Only 10 mg. of Fraction III (not extracted by ethyl acetate or by butanol) were found. In the counter-current distributions, the ethyl acetate mid-piece, the butanol mid-piece, and the slow moving butanol left end-piece were obtained from the peaks which occurred in the same positions in the series as were found in the case of DNP insulin. The melting points of these compounds were the same as those reported above. Impure colored fractions occurred at both left and right ends in the distribution of both Fractions I and II.

Isolation of Ethyl Acetate Mid-Piece and Butanol Mid-Piece from Digests of DNP Trypsinogen—Crystalline trypsinogen[9] was converted to the DNP protein by the same procedure as that used with insulin (2). DNP trypsinogen, in contrast to DNP insulin, would not dissolve in cold or hot solutions of K_2HPO_4, and in order to bring it into solution, heating to boiling in 0.1 N NaOH for a minute was required. The degradation of the protein by this drastic procedure may have been extensive. As soon as solution was effected, the pH was lowered to 8. 1 gm. of DNP trypsinogen was thus dissolved in 400 cc. of water and digested with 100 mg. of pancreatin at 37° overnight. The solution was buffered with 500 mg. of K_2HPO_4 during this operation. The digest was then acidified to pH 3 and treated in the same manner as that described for DNP insulin. Fraction I (the ethyl acetate extract) yielded an ethyl acetate mid-piece, the position of which in the counter-current distribution series was identical to that for the ethyl acetate mid-piece of insulin. This fraction behaved the same as that from DNP insulin in the counter-current distribution with ethyl acetate and phosphate buffer. The symmetry of the peak of color, found in a counter-current distribution of the purified fraction through fourteen tubes of butanol and dilute aqueous ammonia, was established in the same way as that described for the ethyl acetate mid-piece of DNP insulin. The purified compound from DNP trypsinogen showed the same R_F values as did the corresponding fraction from DNP insulin when tested on paper strips with butanol-ammonia and with phenol-ammonia. When the fraction from DNP trypsinogen was tested on the same paper strip with that from DNP insulin, the two substances were found to move at the same rate. The melting point of the compound isolated from DNP trypsinogen was the same as that described for the ethyl acetate mid-piece from DNP insulin. The yield of purified material was 10.6 mg.

Fraction II (the butanol extract of the digest) was treated in the manner described for the corresponding fraction from DNP insulin. A peak of color appeared in the same position in the counter-current distribution as that occupied by the butanol mid-piece from DNP insulin. This colored fraction was purified by counter-current distribution between butanol and phosphate buffer and it was found to behave exactly as did the butanol mid-piece from DNP insulin. The purified substance from the butanol-phosphate distribution was distributed counter-currently between butanol and dilute aqueous ammonia through twenty tubes, and it appeared as a single colored peak with a maximum in Tube 10. The symmetry of this peak was demonstrated by secondary counter-current distributions, as have been described above. The purified substance was tested on paper strip chromatograms and was found to have the same R_F values in butanol-ammonia and in phenol-ammonia as those described for the butanol mid-piece from DNP insulin. These determinations were carried out simultaneously with those for the substance from insulin. The melting point of the compound from DNP trypsinogen was the same as that described for the butanol mid-piece from DNP insulin. The yield was 5.5 mg.

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[9] Crystalline trypsinogen very kindly supplied by Dr. M. Kunitz of this Institute.

Isolation of New DNP Peptide from DNP Trypsinogen—When Fraction I (ethyl acetate extract) of the DNP trypsinogen digest was distributed counter-currently between butanol and dilute aqueous ammonia, a new colored substance appeared which had not been found in the digest of DNP insulin. This substance showed a peak of color with a maximum in Tube 3 of a series of fourteen. Tubes 2, 3, and 4 were combined, the solvent was removed under reduced pressure, and the residue was purified by counter-current distribution through twelve tubes, each containing 7.5 cc. of butanol and 2.5 cc. of 5 per cent aqueous ammonium hydroxide. A symmetrical peak with a maximum in Tube 6 was found. The symmetry of this peak was demonstrated by secondary counter-current distributions such as those described above. The colored substance moved as a single spot on paper strip chromatograms. The R_F of this spot was 0.20 with butanol-ammonia and 0.68 with phenol-ammonia. When the solutions were evaporated, the substance tended to remain oily and crystallized only after prolonged storage. The yield was 4.1 mg.

Amino Acid Composition of DNP Peptides—To determine the qualitative amino acid composition of a fraction, about 1 mg. was refluxed with 15 cc. of 20 per cent HCl for 24 hours, and the hydrolysate was evaporated to dryness under reduced pressure repeatedly, neutralized, and made to 1 cc. 0.02 cc. was analyzed on strips of Whatman No. 1 filter paper according to the directions of Consden, Gordon, and Martin (6). Phenol was the most satisfactory solvent tested, and it was used routinely. Occasionally butanol, or a mixture of butanol and benzyl alcohol, or collidine, was also used. All fractions were examined with phenol saturated with water and with phenol saturated with 3 per cent aqueous ammonium hydroxide. As recommended by the originators of the method, a known mixture of amino acids approximating the composition of the unknown was always run beside the unknown on the same strip of paper.

In the case of fractions judged to be homogeneous (such as the mid-pieces) the results of the paper strip analysis were confirmed by microbiological assays. The qualitative composition of these substances is shown in Table I. The quantitative results of microbiological assay are shown in Table II. The values in Table II serve only to indicate the molecular proportions of amino acids in the hydrolysates and do not represent percentages of these constituents in the peptides. In most cases the amino acids were in equimolecular proportions. However, in the slow moving butanol left end-piece, the ratio of leucine to the other components was about 3:1. Valine, in this peptide, was too high for a 1:1 ratio. Aspartic acid, isoleucine, and threonine were determined with the aid of *Leuconostoc mesenteroides* and glutamic acid, leucine, and valine with *Lactobacillus arabinosus*. Threonine was also determined with *Streptococcus faecalis*. The same basic amino acid composition in the medium (8, 9) was used in all instances, and the particular acid to be determined was omitted for the assay. A micro modification of the usual procedures

TABLE I

Qualitative Amino Acid Composition of Purified DNP Peptides as Judged by Paper Strip Chromatography and by Microbiological Assay

Description of fraction	Chromophore	Glutamic acid	Serine	Threonine	Alanine	Leucine	Isoleucine	Valine	Aspartic acid	Cystine
From DNP insulin										
1. Ethyl acetate left end-piece	+	+								
2. " " mid-piece	+	+	+	+	+	+	+	+		
3. Butanol mid-piece	+	+	+	+	+	+	+	+	+	
4. Slow moving butanol left end-piece	+	+	+	+		+	+	+	+	
5. Butanol left end-piece	+	+	+		+	+	+	+	+	+
6. HCl cleavage product of (4)	+	+				+	+			
7. " " " " (3)*†	+	+								
From DNP trypsinogen										
8. Ethyl acetate mid-piece	+	+	+	+	+	+	+	+		
9. Butanol mid-piece	+	+	+	+	+	+	+	+	+	
10. Ethyl acetate slow piece*	+		+				+			

* Traces of the other amino acids contained in Peptide 3 could be detected if a high concentration of hydrolysate was tested.

† Not the same as Peptide 1 because it moved at a faster rate in counter-current distribution (*cf.* the text).

was employed in which a total volume of 1 cc. of medium was used per tube.

Nature of Chromophoric Group. (a) Counter-Current Distribution of Synthetic DNP Compounds—2 mg. samples of various synthetic DNP compounds were distributed counter-currently between butanol and dilute aqueous ammonia through a series of eight tubes. 5 cc. of each solvent were used in each tube. The characteristic rate of migration of each of the compounds tested is shown in Table III. Values for other solvent systems are also given.

Table II

Amounts of Various Amino Acids Found in Hydrolysates of DNP Peptides

The results are in micrograms per cc. of hydrolysate and do not represent percentages in the peptides.

Peptides	Aspartic acid	Glutamic acid	Threonine	Valine	Leucine	Isoleucine
From DNP insulin						
1. Ethyl acetate mid-piece	0	50	42	39	46	52
2. Butanol mid-piece		150		124	140	120
3. Slow moving butanol left end-piece	25	25	19	28	68	
4. Butanol left end-piece		85		78	72	80
5. HCl cleavage product from (3)		70			60	60
From DNP trypsinogen						
6. Ethyl acetate mid-piece	0		24	35	37	
7. Butanol mid-piece			73	90	101	

(b) Isolation of DNP-Glycine and DNP-Phenylalanine from DNP Insulin—50 mg. of DNP insulin were refluxed in 15 cc. of 20 per cent HCl for 4 hours, and the acid was removed under reduced pressure. The residue was suspended in water, and the suspension was extracted four times with ethyl acetate. The extract was freed of solvent and partitioned counter-currently between butanol and dilute aqueous ammonia through eight tubes with 5 cc. of each solvent in each tube. Maxima of color, or peaks, appeared in Tubes 3, 6, and 8. The contents of Tubes 2 and 3 were freed of solvents and counter-currently distributed between ethyl acetate and 0.1 M acetate buffer made from 1 volume of acetic acid and 3 volumes of sodium acetate. When the distribution was continued through eight tubes, a good peak was found in Tube 4. The contents of Tubes 3 to 5 were acidified, and the ethyl acetate layers were evaporated. The residue was washed with water, dried, and shown to be DNP-glycine by its melting point of 205°. DNP-phenylalanine was obtained from Tubes 6 and 7 of

TABLE III

Position of Various Synthetic Compounds in Counter-Current Distribution between Immiscible Solvents in Series of Eight Tubes with Equal Volumes in Both Layers

Compound	Tube No., showing maximal color			
	With butanol and aqueous NH_4OH*	With ethyl acetate-acetate buffer†	With ethyl acetate-phosphate buffer‡	
			2:1 Na_2H-NaH_2	1:2 Na_2H-NaH_2
DNP-glycine	3	4	1	
DNP-phenylalanine	6	8	4	
2,4-Dinitroanaline	8			
2,4-Dinitrophenol	5			4
DNP-methionine	5	7	1	3
DNP-alanine	4	6	1	1
DNP-serine	3			
DNP-threonine	3	3		
DNP-proline	4, 5	6	1	1
DNP-hydroxyproline	3			
DNP-valine	7			
DNP-leucine	6			
DNP-isoleucine	6			
DNP-aspartic acid	1			
DNP-glutamic acid	1			
Di-DNP-tyrosine	8			
Di-DNP-lysine§	8	8		
ε-DNP-lysine‖	5			
DNP-arginine	5, 6			1
DNP-histidine	6, 7			
DNP-cystine	3			
DNP-glycyldehydroalanine	1**	1		
Bis-DNP-diglycyl-α-aminoalanine	4			1

* 95 cc. of water + 5 cc. of concentrated ammonium hydroxide.

† 1 volume of 0.1 M acetic acid + 3 volumes of 0.1 M sodium acetate.

‡ Buffer made by mixing 0.1 M solutions of Na_2HPO_4 and of NaH_2PO_4 in the proportions indicated.

§ In a system composed of 5 per cent aqueous ammonium hydroxide as the bottom layer and 2:1 ethyl acetate-ether as the top layer, the peak was in Tube 3. With just ethyl acetate as the top layer the peak was in Tube 6. The sodium salt was completely extractable from aqueous solution with ethyl acetate.

‖ In a system of butanol and 0.1 N HCl, the peak was in Tube 6.

** The peak was in Tube 2 when 3 volumes of butanol to 1 volume of ammonia were used instead of the 1:1 ratio.

the butanol counter-current distribution by a partition with ethyl acetate and 0.1 M phosphate buffer made from 2 volumes of Na_2HPO_4 and 1 volume of NaH_2PO_4.

(*c*) *Demonstration of DNP-Glycine in Fraction III of DNP Insulin Digest*—100 mg. of Fraction III, the part not extractable by ethyl acetate or by butanol, were hydrolyzed with HCl and separated exactly as described for DNP insulin in the preceding paragraph, and DNP-glycine was readily obtained.

(*d*) *Stability of DNP-Glycine to Hot, 20 Per Cent HCl*—5 mg. of DNP-glycine were refluxed in 15 cc. of 20 per cent HCl for 24 hours, and the reaction mixture was concentrated to dryness under reduced pressure. The residue was distributed counter-currently between butanol and dilute ammonia through eight tubes. Most color was found in Tube 3, but a considerable amount of orange color was in Tube 8 (*cf.* (2)). Thus, some decomposition had occurred during the prolonged acid treatment.

(*e*) *Investigation of Chromophoric Group of DNP Peptides Isolated from DNP Insulin Digests*—Samples of 10 to 30 mg. of each of the compounds were refluxed in 15 cc. of 20 per cent HCl for 4 hours, and the acid was removed by evaporation under reduced pressure. The residue was then taken up in water, and the suspension which resulted was extracted five times with ethyl acetate. The extract was freed of solvent and counter-currently distributed between butanol and dilute aqueous ammonia. In every case, the major part of the color was divided into two components, one of which remained mostly in Tube 1 and the other in Tube 8. In the case of the butanol mid-piece, the butanol right end-piece, and the ethyl acetate mid-piece, a faint peak was discernible in Tube 3, which should represent DNP-glycine. Therefore, the contents of Tubes 2 to 4 were freed of solvents under reduced pressure and counter-currently distributed between ethyl acetate and 0.1 M acetate buffer (made from 3 volumes of sodium acetate and 1 of acetic acid). When the distribution was continued through eight tubes, a peak characteristic for DNP-glycine appeared in Tube 4. However, the amount of color thus to be attributed to DNP-glycine was only a small fraction of the total in the hydrolysate. The major colored hydrolysis product was found in the right-hand tube of the butanol-ammonia counter-current series, and this was shown to be a neutral substance because it could be extracted from either strongly acidic or alkaline solutions into ether. Results practically identical with these were found when the period of hydrolysis was 20 hours instead of 4.

There was some evidence for another colored component in the hydrolysates because the acidic aqueous phase which remained after the ethyl acetate extraction was still brownish yellow. It was not known whether this color arose from decomposition during hydrolysis or whether it was due to some yellow constituent not extractable with ethyl acetate. It was noted particularly in hydrolysates of the mid-pieces. On a paper strip chromatogram with butanol and ammonia this color moved at the same rate as did ϵ-DNP-lysine.

(*f*) *Anomalous Behavior of Bis-DNP-diglycyl-α-aminoalanine on Acid Hydrolysis*—Diglycyl-α-aminoalanine (10) was converted to the DNP derivative according to the general procedure described by Sanger for DNP amino acids, and the bis-DNP-diglycyl-α-aminoalanine was purified by counter-current distribution between butanol and dilute aqueous ammonia (*cf.* Table III). It was finally obtained as the water-insoluble free acid which melted at 170–175°.

$C_{19}H_{18}O_{12}N_8 \cdot H_2O$. Calculated, N 19.8; found, N 19.5

10 mg. were refluxed for 4 hours in 20 per cent HCl, and the product was fractionated counter-currently with butanol and dilute ammonia. No trace of starting material or of DNP-glycine was found, but instead, two colored fractions, a left end-piece and a right end-piece, were present.

By treatment with cold, concentrated HCl, followed by separation and hydrolysis of the individual cleavage products, DNP-glycine could be isolated from the compound, thus leaving little doubt that it was actually contained in the molecule. Cold, concentrated HCl (0.5 cc.) in 4 hours split the compound (5 mg.) quantitatively into two colored substances which were separated by counter-current distribution between butanol and aqueous ammonia. One product was identified as DNP-glycyldehydroalanine, found in Tubes 1 and 2 of the counter-current series,[10] and the other was a neutral compound, found in Tube 8, with the melting point of DNP-glycine amide (245°). When either one of these products was refluxed alone in 20 per cent HCl, DNP-glycine was formed, which was isolated by methods already described.

(*g*) *DNP-Methionine from Hydrolysate of Ethyl Acetate Slow Piece of DNP Trypsinogen*—4 mg. of the ethyl acetate slow piece from DNP trypsinogen were dissolved in 15 cc. of 20 per cent HCl and the solution was refluxed for 24 hours. Excess HCl was removed under reduced pressure, the residue was suspended in water, and the suspension was extracted three times with ethyl acetate. The extracts were freed of solvent under reduced pressure and the yellow substance was distributed counter-currently, first in butanol and dilute aqueous ammonia, then in ethyl acetate-acetate buffer, and finally in ethyl acetate and phosphate buffer. The compositions of these buffers were those shown in Table III. The yellow material behaved in each of the solvent systems as did DNP-methionine (*cf.* Table III). The quantity of material available was not sufficient for identification by classical means. In addition to the yellow substance which behaved as DNP-methionine, there was also some orange material which remained in the right-hand end tube of the butanol-ammonia distribution.

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[10] If care was exercised to prevent the temperature from rising during any of the operations, an intermediate compound could be distinguished as a colored peak in Tube 2 of a series of eight counter-current tubes. This was probably DNP-glycyl-α-hydroxyalanine. Gentle warming such as that which occurred during concentration under reduced pressure resulted in the disappearance of this material and the formation of a compound which behaved in a counter-current distribution as did synthetic DNP-glycyldehydroalanine. The course of the hydrolysis, therefore, was the cleavage to DNP-glycine amide by reaction of 1 molecule of water, and the subsequent dehydration of the relatively unstable derivative of α-hydroxyalanine. Synthetic glycyldehydroalanine used for the preparation of the DNP derivative was kindly supplied by Dr. J. P. Greenstein.

Partial Hydrolysis with Cold, Concentrated HCl of Slow Moving Butanol Left End-Piece—25 mg. of the slow moving butanol left end-piece were dissolved in 5 cc. of concentrated HCl for 5 hours at room temperature and the solution was then diluted with 25 cc. of water and extracted twice with butanol. The extracts were treated with 10 cc. of dilute aqueous ammonia, concentrated under reduced pressure to dryness, and the residue was distributed counter-currently between butanol and dilute aqueous ammonia through a series of eight tubes. Peaks of color appeared in Tubes 1, 3, and 8. Contents of Tubes 2, 3, and 4 were freed of solvents under reduced pressure and the residue was distributed counter-currently between butanol and dilute aqueous ammonia through twelve tubes. 7.5 cc. of butanol and 2.5 cc. of aqueous phase were used in each tube. The compound which was now found in Tubes 3, 4, and 5 was obtained as a small amount of crystalline material by evaporation of the solvents. Hydrolysis and analysis showed the presence of only glutamic acid, leucine, and isoleucine.

Partial Hydrolysis with Cold, Concentrated HCl of Butanol Mid-Piece—10 mg. of the butanol mid-piece from DNP insulin were treated with HCl in the manner described in the preceding section. In the counter-current separation, the major part of the color appeared as a peak in Tube 8 of a series of fourteen. When the solutions containing this colored material were evaporated and the residue hydrolyzed, glutamic acid was found as the major amino acid. Traces of the other amino acids derivable from the butanol mid-piece could be detected if relatively high concentrations of the hydrolysate were tested. Because the cleavage product appeared in the same position in a counter-current distribution as did the butanol mid-piece from which it was made, this contamination was impossible to avoid. A similar cleavage product of the butanol mid-piece of DNP trypsinogen was found.

SUMMARY

Methods have been described for the separation of dinitrophenyl amino acids (DNP amino acids) and of DNP peptides. These methods depend on differential extraction and on counter-current distribution of various salts of the DNP compounds.

Pancreatin digests of DNP insulin have been fractionated by these methods to yield four crystalline yellow compounds. These same four crystalline compounds were also obtained from pancreatin digests of the DNP derivative of that fraction of performic acid-oxidized insulin which was soluble at pH 6. Evidence for the homogeneity of these products was presented.

Two compounds indistinguishable in all respects from two of those from DNP insulin were also isolated from digests of DNP trypsinogen. In addition a third substance, not obtainable from DNP insulin, was isolated from DNP trypsinogen and found to yield DNP-methionine, serine, and isoleucine when it was hydrolyzed.

The amino acid composition of each of the substances from DNP insulin was examined, and they were found to be of differing complexity. One contained the chromophoric group and glutamic acid; the second yielded the chromophoric group, glutamic acid, serine, threonine, alanine, valine, leucine, and isoleucine; a third gave these constituents plus aspartic acid; and a fourth contained the same amino acids (except alanine) as the third but differed from it in the order of their arrangement. A fifth substance, the purity of which was not established, was found to contain the chromophoric group, aspartic acid, glutamic acid, serine, alanine, valine, leucine, isoleucine, and cystine. The regular increase in complexity suggested that the simpler ones were degradation products of the more elaborate ones. Some evidence against this view was presented. Although some of the smaller peptides could be derived from one of the largest, another one of the largest molecules apparently had its amino acid residues arranged differently. The amino acid residues occurred in these compounds in equimolecular proportions, except for one in which approximately 3 moles of leucine were present per mole of other constituents.

The chromophoric group was considered to be DNP-glycine, but because of the low yield of this substance which could be isolated from the hydrolysates of the peptides, there was much doubt about this conclusion. Most of the colored material, or chromophore which was liberated by acid hydrolysis, could not be identified with previously described yellow products from DNP insulin. A model compound, bis-DNP-diglycyl-α-aminoalanine, was synthesized and found to behave on hydrolysis with respect to its chromophoric groups much as did the peptides from insulin.

Partial hydrolysis of the larger of the DNP peptides gave rise to smaller yellow compounds and these were isolated. From one of the large peptides the cleavage product was shown to contain the chromophore, leucine, isoleucine, and glutamic acid. From another of the larger peptides the partial cleavage product was composed principally of the chromophore and glutamic acid.

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METABOLISM OF DEHYDROISOANDROSTERONE IN A WOMAN BEFORE AND AFTER REMOVAL OF AN ADRENOCORTICAL TUMOR

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In earlier communications (Mason and Kepler (1, 2)) it was shown in three instances that dehydroisoandrosterone was converted largely to androsterone and etiocholanolone before excretion in the urine. In two cases dehydroisoandrosterone could not be isolated and in the third case it comprised approximately 21 per cent of the crystalline substances isolated. The surprising efficiency of conversion of dehydroisoandrosterone to other substances raised the question as to why often such large amounts of dehydroisoandrosterone relative to the other 17-ketosteroids are excreted in the urine in cases of adrenal cortical tumor. A rather obvious suggestion was that perhaps a large production of dehydroisoandrosterone (or its precursor) by the tumor overwhelmed the mechanisms for its conversion and destruction and led to an increased proportion of dehydroisoandrosterone and therefore of the 3(β)-hydroxyketosteroids (the β fraction) in the urine. It was proposed to test this suggestion by administration of dehydroisoandrosterone to a patient with an adrenal tumor who was already excreting fairly large amounts of 17-ketosteroids with a high proportion of 3(β)-hydroxyketosteroids. If the suggestion had any merit, it was anticipated that a large proportion of the dehydroisoandrosterone administered would be excreted unchanged.

A suitable patient, who consented to be the subject of the study, was a woman twenty-nine years of age, who had become extremely virilized. Urine was collected for 10 days; then 50 mg. of dehydroisoandrosterone acetate in sesame oil[1] were injected three times daily (equivalent to a total of 131 mg. of dehydroisoandrosterone) for 8 days. All of the urine was collected during this period and for 3 days afterward, and pooled. Urine during the next 4 days was collected separately for isolation of the steroids. Previous experience indicated that the steroid content of the urine collected at this time would not be influenced by the injections of dehydroisoandrosterone acetate, and misfortune occurred in the isolation of steroids from the urine collected during the pretreatment period (first control period).

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* Died October 19, 1947.

[1] We are indebted to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for this preparation.

After the patient had recovered from the operation for removal of an adrenal cortical tumor, beginning on the 24th postoperative day, dehydroisoandrosterone acetate again was administered in the same manner and amount. The amount of 17-ketosteroids excreted during the postoperative control period was so small that no attempt to isolate steroids from the urine collected during this period was made. Also for this reason determinations of the β fraction were not made. Determinations of the total 17-ketosteroids and of the β fractions were made during the first control period and daily during both periods in which the patient received dehydroisoandrosterone acetate.

TABLE I

Excretion of 17-Ketosteroids during Administration of Dehydroisoandrosterone before Operation

Day No.	Total 17-ketosteroids	β fraction
Control period		
	mg. per 24 hrs.	*per cent*
1	271	45
2	256	33
3	248	23
50 mg. dehydroisoandrosterone acetate 3 times daily		
4	252	28
5	226	23
6	319	32
7	285	37
8	262	42
9	256	47
10	296	36
11	296	44
After period		
12	272	46
13	236	24
14	256	48

The results of the quantitative determinations are given in Tables I and II. The average value for the total 17-ketosteroids during the first control period (Table I) was 258 mg. (range 248 to 271) in 24 hours, 23 to 45 per cent of which was the β fraction. On the 7th and 8th days of administration of dehydroisoandrosterone acetate the value of the total 17-

ketosteroids had increased to 296 mg. in 24 hours. The average value for the 8 days, however, was 274 mg., with a range of 252 to 296 mg. It seems likely that the collections of urine on Days 5 and 6 were not accurate, since the low value on Day 5 was compensated by the high value on Day 6. Thus, on the average, the daily administration of 150 mg. of dehydroisoandrosterone acetate caused an increase of only 16 mg. in the daily amount of 17-ketosteroids excreted. The β fraction varied from 23 to 47 per cent of the total amount and never increased significantly beyond the maximal value obtained during the control period or the after period. It is evident that the daily addition of 150 mg. of dehydroisoandrosterone acetate to the steroids being metabolized did not produce a significant increase in the amount of 17-ketosteroids excreted or in the proportion of the β fraction.

TABLE II

Excretion of 17-Ketosteroids during Administration of Dehydroisoandrosterone after Removal of Adrenal Tumor

Day No.	Total 17-ketosteroids	β fraction
Control period		
	mg. per 24 hrs.	*per cent*
1	2.8	
2	2.5	
3	2.5	
4	3.3	
50 mg. dehydroisoandrosterone acetate 3 times daily		
5	15.1	6
6	40.3	8
7	54.4	23
8	57.5	13
9	59.4	14
10	58.8	24
11	31.7	38
12	52.2	36

After removal of the tumor the excretion of 17-ketosteroids was only 2.5 to 3.3 mg. per day (Table II). Injection of 150 mg. of dehydroisoandrosterone acetate daily for 8 days increased the excretion of 17-ketosteroids to between 50 and 60 mg. after the 2nd day. There was also a progressive increase in the β fraction to a maximum of 38 per cent in the 8 day period.

The results indicate that before operation the added dehydroisoandrosterone was very efficiently metabolized to substances not recognizable as 17-ketosteroids. Dehydroisoandrosterone did not displace other 17-ketosteroids in the urine, since the β fraction was not increased. After operation, however, excluding the 1st day, the average amount of 17-ketosteroids attributable to dehydroisoandrosterone was 47.8 mg. (50.6 mg. minus the average control value, 2.8 mg.), or approximately 36 per cent of the amount given. Furthermore, an appreciable proportion was in the β fraction, which presumably was largely unchanged dehydroisoandrosterone (see the results of the isolation).

The results of the isolation of the urinary steroids are summarized in Tables III and IV. A summary of the crude fractions is given in Table III, and of the crystalline fractions in Table IV. Unfortunately the extract of the urine collected before the first administration of dehydroisoandrosterone was lost. However, the urine, collected just prior to operation, on the 4th, 5th, 6th, and 7th days after administration of dehydroisoandrosterone acetate had been stopped served as a control specimen.

TABLE III

Crude Fractions Isolated

Fraction	Preoperative control period, 4 days	Preoperative period, DHA* given, 11 days†	Postoperative period, DHA* given, 8 days
	gm.	*gm.*	*gm.*
Neutral	1.823	4.575	0.979
Ketonic	1.107‡	2.621	0.452
Non-ketonic	0.484	1.351	0.428
Alcoholic ketonic		1.652	0.354
" non-ketonic	0.258	0.657	0.087
Non-alcoholic ketonic		0.395	0.061
" non-ketonic	0.071	0.318	0.416

* Dehydroisoandrosterone acetate.

† Urine collected for 3 days after administration of DHA was stopped was included in this period.

‡ This ketonic fraction was chromatographed without further fractionation.

In accord with the quantitative determinations of the β fraction, dehydroisoandrosterone, together with its derivative, chlorodehydroandrosterone, comprised more than half of the isolated ketones during the two preoperative periods. This relation is in agreement with previous observations that the excretion of dehydroisoandrosterone usually is increased greatly in cases of adrenal cortical tumor. There appear to be no significant qualitative or quantitative differences in the steroids isolated in the two preoperative periods. In the period after removal of the tumor there is evidence of conversion of dehydroisoandrosterone to androsterone and etiocholanolone, this time in a person who was not suffering from Addison's disease or pituitary insufficiency. Also, a relatively large amount of unchanged dehydroisoandrosterone was recovered, 13.7 per cent of the amount administered and 65.8 per cent of the ketones isolated. A much smaller proportion of dehydroisoandrosterone was recovered in the three cases previously described; indeed, none could be isolated in two of these cases.

The weights given for androsterone isolated during the periods when the tumor was present may be misleading, since Lieberman and associates (3) have shown that this fraction may contain large amounts of Δ^9-androsten-3(α)-ol-17-one, which cannot be separated readily from androsterone and which do not depress the melting point of the latter. Although we have been able in almost every case of adrenal tumor to isolate 11(β)-hydroxyandrosterone, the precursor of the Δ^9-androstenolone, it was not possible to isolate it in this case.

TABLE IV

Steroids Isolated

Compound	Preoperative control period, 4 days		Preoperative period, 1200 mg. DHA* given, 11 days†		Postoperative period, 1200 mg. DHA* given, 8 days	
	total mg.	*mg. per day*	*total mg.*	*mg. per day*	*total mg.*	*mg. per day*
Androsterone	72	18.0	200	18.2	43	5.4
Dehydroisoandrosterone	232	58.0	488	44.4	144	18.0
Etiocholan-3(α)-ol-17-one	78	19.5	283	25.7	32	4.0
3-Chlorodehydroandrosterone	18	4.5	51	4.6		
Δ^5-Androstene-3(β),17(α)-diol	5	1.2	14	1.3		
Pregnane-3(α),20(α)-diol	10	2.5	82	7.5	18	2.2
Alcohol, $C_{21}H_{34}O$	10	2.5	80	7.3		

* Dehydroisoandrosterone acetate; 1200 mg. are equivalent to 1047 mg. of free dehydroisoandrosterone.

† Urine collected for 3 days after administration of DHA was stopped was included in this period.

The alcohol, $C_{21}H_{34}O$, was encountered in another case of adrenal tumor in relatively large amounts in the fraction conjugated with glucuronic acid. Its identity remains unknown. However, it formed a monoacetate and benzoate, and took up 2 hydrogen atoms in the presence of platinum catalyst. Presumably it is a pregnenol. The chemistry of this substance will be the subject of a later communication.

Δ^5-Androstene-3(β),17(α)-diol[2] has been shown to be a metabolite of dehydroisoandrosterone. Δ^5-Androstene-3(β),16(β),17(α)-triol[2] was not found, although it was sought exhaustively. When the tumor was present, the significance of the presence of pregnanediol is uncertain, since this patient had regular cyclic periods of bleeding which simulated normal menstruation. Four other alcoholic non-ketones were obtained in amounts too small for further investigation.

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[2] Although recent evidence indicates that the configuration in these two compounds should be 17(β) and 16(α),17(β), respectively, they are here designated as in the preceding papers.

Comment

Obviously the results in this one case do not support the hypothesis stated at the beginning of this paper. Since we have no measure of the amount of dehydroisoandrosterone (or its precursors) being produced by the tumor and metabolized, it may be that the amount of this substance injected was insignificant in comparison with the total amount being metabolized in various ways. However, if the mechanisms for metabolism of dehydroisoandrosterone were saturated, thus allowing a portion to escape into the urine, it would be expected that any additional amount would appear in the urine. Actually, it seems that the mechanisms for disposal of dehydroisoandrosterone were more effective in the presence of the tumor than after its removal.

Previous studies (Mason and Kepler (1, 2)) on the metabolism of dehydroisoandrosterone were made with subjects having pituitary insufficiency and adrenal cortical insufficiency. These subjects were, of course, not metabolically normal, but were selected because their endogenous production of steroid hormones was virtually nil. The question has arisen as to what effect their general metabolic deficiencies would have on the metabolism of dehydroisoandrosterone and other steroids. In the present case it may be argued that, when dehydroisoandrosterone was administered after operation, sufficient time had not elapsed to permit the patient to recover fully from the effects of the operation or of the prior stimulus of large amounts of adrenal cortical hormones. Nevertheless, the patient had a functioning adrenal cortex and was metabolically normal, as far as could be determined. Since the metabolism of dehydroisoandrosterone was similar to that previously found, these results add further support to the conclusion that dehydroisoandrosterone is normally converted largely into androsterone and etiocholanolone before excretion in the urine. Individual quantitative differences have been pointed out previously.

The daily excretion of 17-ketosteroids was somewhat low (2.5 to 3.3 mg.) at the time of the study after operation. However, a single determination 10 months after operation gave a value of 5.5 mg. per 24 hours and another 19 months after operation gave a value of 4.3 mg.

EXPERIMENTAL

The 17-ketosteroids were determined in an aliquot of each daily specimen of urine by the method of Callow, Callow, and Emmens (4). The readings were corrected by the equation of Talbot, Berman, and MacLachlan (5). The β fraction was determined essentially by the method of Talbot, Butler, and MacLachlan (6). However, after precipitation of the digitonides overnight in the refrigerator and centrifugation, purified cholesterol was added to precipitate the excess of digitonin. The 17-ketosteroids not precipitated by digitonin were determined in the supernatant and the β fraction was calculated. The total 17-ketosteroids were determined in an aliquot of the original extract treated in the same way, except for omission of the digitonin, in order to compensate for any changes of volume during the rather long procedure.

The urine remaining after determination of the 17-ketosteroids was pooled and processed in the manner previously described (7). The neutral extract was separated into alcoholic ketonic, non-alcoholic ketonic, and non-alcoholic non-ketonic fractions with the aid of Girard's Reagent T and succinic anhydride. The last fraction was discarded. Individual compounds were separated by chromatographic analysis on columns of aluminum oxide (Fisher's alumina for chromatographic analysis) by using carbon tetrachloride (for ketonic fractions) and benzene (for non-ketonic fractions) containing increasing small amounts of alcohol.

The major part of the dehydroisoandrosterone was isolated as the hemisuccinate by crystallization from acetone of the mixed hemisuccinates of the alcoholic ketonic fraction. After hydrolysis of the hemisuccinates in the mother liquor, the remaining dehydroisoandrosterone was precipitated with digitonin before this fraction was subjected to chromatographic analysis. Dehydroisoandrosterone, androsterone, and etiocholanolone were identified by comparison with authentic specimens and by comparison of the corresponding acetates. Δ^5-Androstene-3(β),17(α)-diol and pregnane-3(α),20(α)-diol were identified in a similar manner. Chlorodehydroandrosterone (m.p. 155–156°) was identified by a positive Beilstein test and by comparison with an authentic specimen.

Alcohol, $C_{21}H_{34}O$—This substance was eluted from the column of alumina by benzene alone when the alcoholic non-ketonic fraction was chromatographed. The crystalline material weighed 80 mg. and melted at 140–141°. It formed an acetate which melted at 131–132°. The alcohol proved to be identical with a fraction obtained in another case of adrenal cortical tumor from the conjugates hydrolyzed with glucuronidase. In this latter case the empirical formula was established by analysis of the alcohol and the acetate. This compound will be the subject of a further communication. At this time it appears to be a pregnenol.

SUMMARY

Dehydroisoandrosterone acetate (150 mg. per day) was administered to a woman with an adrenal cortical tumor who was excreting an average of

258 mg. of 17-ketosteroids per day. This amount of extra 17-ketosteroid resulted in an average increase in the excretion of 17-ketosteroids of only 16 mg. There was no detectable change in the amount of the β fraction. After removal of the tumor, the daily administration of 150 mg. of dehydroisoandrosterone acetate resulted in an average increase of 47.8 mg. in the amount of 17-ketosteroids excreted and the β fraction increased to 38 per cent of the total.

During the periods without treatment and with treatment with dehydroisoandrosterone acetate while the tumor was present, androsterone, dehydroisoandrosterone, etiocholan-3(α)-ol-17-one, 3-chlorodehydroandrosterone, Δ^5-androstene-3(β),17(α)-diol, pregnane-3(α),20(α)-diol, and an alcohol, $C_{21}H_{34}O$, probably a pregnenol, were isolated from the urine. During the period of administration of dehydroisoandrosterone acetate after removal of the tumor, androsterone, dehydroisoandrosterone, etiocholan-3(α)-ol-17-one, and pregnane-3(α),20(α)-diol were isolated. The isolation of androsterone and etiocholanolone in this last period is further evidence for the metabolic conversion of dehydroisoandrosterone to these substances, although the extent of the conversion was not as great as was found previously in two cases of Addison's disease and one of pituitary insufficiency.

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THE VERATRINE ALKALOIDS

XXIX. THE STRUCTURE OF RUBIJERVINE

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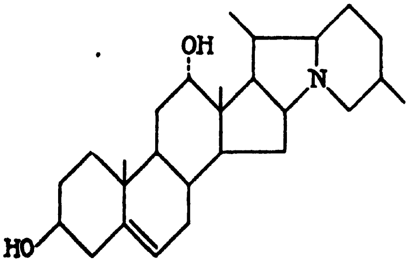
Rubijervine has been shown to be a tertiary steroid base of 3(β)-hydroxy-Δ^5-stenol character. This was supported by its formulation, $C_{27}H_{43}O_2N$, by the formation of a digitonide, by its hydrogenation to a dihydro derivative, and by its oxidation to a Δ^4-ketone, which in turn could be reduced to epimeric 3(α)- and 3(β)-Δ^4-stenols which gave characteristic Rosenheim reactions (1-3). The presence of a second hydroxyl group was shown by the formation of a basic diacetyl derivative (2). On dehydrogenation with selenium, rubijervine yielded the characteristic 2-ethyl-5-methylpyridine obtained from other veratrine alkaloids (4-6) and from solanidine (2, 7). Contrary to the latter, however, as in the case of the other veratrine bases, no Diels' hydrocarbon was isolated. Instead, an isomeric methylcyclopentenophenanthrene (1), not previously encountered, was obtained which has not been identified with certainty but appeared to agree in properties with synthetic α-methyl-1,2-cyclopentenophenanthrene (8). In addition an appreciable amount of a phenolic hydrocarbon was obtained for which the formulation, $C_{18}H_{16}O$, was derived and which appeared to be a derivative of a methylcyclopentenophenanthrene (1). This dehydrogenation product was not isolated in the case of any of the other bases and thus far is presumably a product characteristic of rubijervine and probably due to its extra hydroxyl group. More recently in work undertaken to remove this hydroxyl it has been definitely shown, by its conversion into solanidine and into solanidanol-(3β) (9), that rubijervine is a hydroxysolanidine. From the general evidence, the most satisfactory interpretation for the structure of rubijervine, barring possible epimerizations, appears to be a 12(α)-hydroxysolanidine (Δ^5-solanidene-3(β), 12(α)-diol), Formula I.



I

A number of preliminary attempts to prepare rubijervine monoacetate resulted in the preponderant formation of the diacetate, and this was abandoned in favor of benzoylation when the latter proved more satisfactory. On benzoylation rubijervine yielded varying proportions, depending upon the conditions used, of *rubijervine-3-benzoate* and *rubijervine dibenzoate* which could be separated chromatographically. That the 3-hydroxyl group was more readily benzoylated with the initial preferential formation of the 3-benzoyl derivative was shown by the failure of the monobenzoate to form a digitonide, and by the fact that its protection led to the production of solanidine. Oxidation of the monobenzoate with chromic acid yielded the *monoketobenzoate* (*Δ^5-solanidene-3(β)-ol-12-one benzoate*), which could be readily saponified to *rubijervone-12* (*Δ^5-solanidene-3(β)-ol-12-one*). The benzoate, however, was used for conversion into the *semicarbazone*, which in turn was reduced by the Wolff-Kishner method. The reaction product, after purification through alumina, yielded a base which agreed in all properties such as melting point and rotation with solanidine. This was confirmed by the comparison of its acetyl derivative with acetylsolanidine.

In the conversion of rubijervine into solanidanol-(3β), dihydrorubijervine (2) was oxidized with chromic acid to the *diketo derivative* (*solanidane-3,12-dione*). The *disemicarbazone* of the latter, when subjected to the Wolff-Kishner reduction, yielded a mixture from which solanidanol-(3β) was isolated in fair yield. Along with the latter a small amount of a by-product was obtained which was believed to be solanidane. The anomalous behavior of the 3-semicarbazone group, in that it was reduced to the hydroxyl group, is in agreement with the observations made by Dutcher and Wintersteiner (10). These experiments, barring rearrangement, have shown that rubijervine must possess the same configuration as solanidine but with an extra secondary hydroxyl group.

In the course of the work *dihydrorubijervine dibenzoate* and *dihydrorubijervine-3-benzoate* were also prepared. The latter was oxidized to *solanidane-3(β)-ol-12-one benzoate*, and unsuccessful preliminary attempts were made to dehydrogenate this substance partially to a possible $\Delta^{9(11)}$-12-keto derivative with selenious acid in accordance with the procedure of Schwenk and Stahl (11).

In an effort to locate the position of the extra secondary hydroxyl group, a series of studies was made. The ready formation of a monoacyl derivative and the lack of ring cleavage on mild oxidation seemed to exclude positions 2 and 4, vicinal to the 3(OH) group. Oxidation with periodic acid was nevertheless attempted on rubijervine but, as expected, no reaction was observed. The stability of the diketone observed with alkali, its absorption spectrum (Fig. 1), and the non-formation of a pyridazine derivative (12) with hydrazine also preclude the presence of the OH group on position 1.

The stability of rubijervine towards acids and the apparent lack of an α,β-unsaturated carbonyl group in the 3-hydroxyketo compound (Δ^5-solanidene-3(β)-ol-12-one), as shown by the absorption spectrum (Fig. 1), makes assignment of OH to position 7 likewise untenable. In view of the accepted inactivity of the carbonyl group at position 11 towards ketonic reagents (13), this position can also be eliminated, since the diketodihydro derivative (solanidane-3,12-dione) forms the disemicarbazone. This leaves as possibilities only position 12 in Ring C, position 15 in Ring D, and position 23 or 24 in the heterocyclic portion (position 26 is eliminated because such a carbonyl derivative would be a substituted neutral lactam).

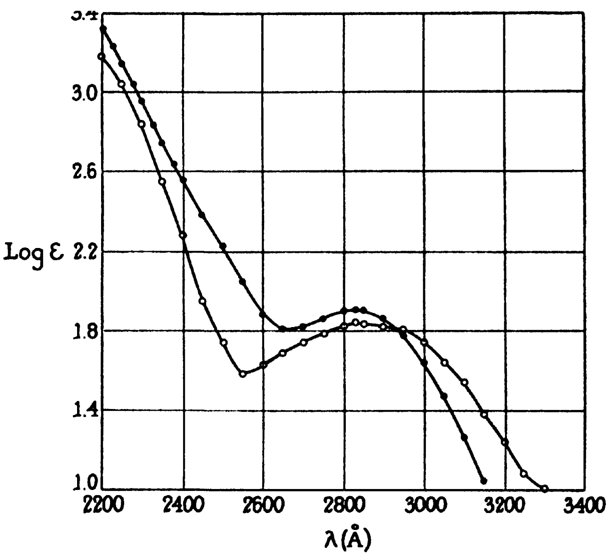


FIG. 1. ●, Δ^5-solanidene-3(β)-ol-12-one; ○, solanidane-3,12-dione; both in ethanol

Whereas solanidine yields Diels' hydrocarbon upon selenium dehydrogenation, an isomer was obtained from rubijervine. If this result is due in some way to the interference offered by the second OH group towards the normal shift of the angular methyl group, hydroxyls located on Ring C or D might seem more likely to interfere with such a shift than those on the N ring. In such a case, positions 12 and 15 would appear to be the most likely for the second hydroxyl group. Some evidence seems to favor the 12 position. Considerable difficulty was encountered in the preparation of the semicarbazone of the ketomonobenzoate and disemicarbazone of the diketodihydro derivative. Analyses of the samples invariably showed a low nitrogen content and only by a purification which involved considerable loss of material could satisfactory analytical figures be obtained. This is somewhat in accord with the findings of Dutcher and Wintersteiner (10) that ketones with carbonyl at position 12 can, on treatment with semicarbazide acetate, lead to incomplete semicarbazone formation.

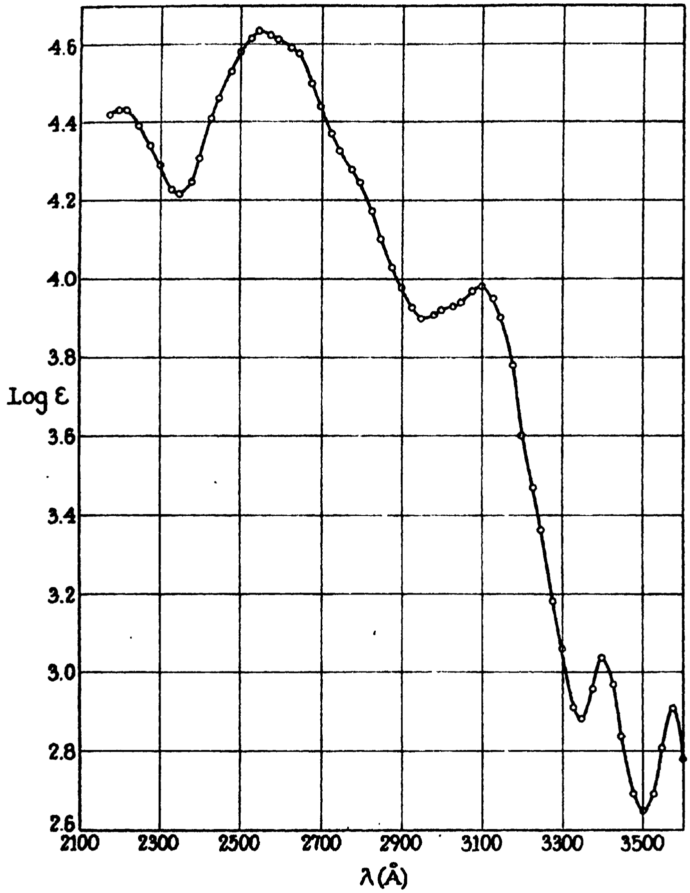


FIG. 2. Absorption curve of $C_{18}H_{16}O$ from rubijervine; in ethanol

Optical rotation data also seem to favor the 12 position on applying Barton's (14) method of molecular rotation differences. The molecular rotation of dihydrorubijervine, $[M]_D$ = +220°, less the rotation of solanidanol-3β) (9), $[M]_D$ = +113°, is +107°. This is in fairly good agreement with the value listed by Barton for a 12(α)OH, *viz.*, +93°. Also the rotation for the diketodihydro derivative, $[M]$ = +489°, less the rotation for solanidane-3-one, $[M]_D$ = +182°, gives +307°, which is compatible with the data given by Barton for the 12-keto group of +270° (14).

Finally, in the phenolic dehydrogenation product of rubijervine $C_{18}H_{16}O$, (ultraviolet absorption shown in Fig. 2) apparently characteristic of the latter, the favored interpretation is that the phenolic group comes from the extra hydroxyl of the base. If so, this would exclude any position on Ring D or the side chain but not position 12. Although the data presented favor such a 12(α)-hydroxyl group, further studies are necessary to make this conclusion final.

EXPERIMENTAL

Rubijervine Monobenzoate and Dibenzoate—In a typical experiment 500 mg. of rubijervine were dissolved in 5 ml. of dry benzene and 5 ml. of dry pyridine by slight warming and 0.3 ml. of benzoyl chloride was added to the solution. This was then heated in an oil bath for 50 minutes at 100° (±5°). Most of the solvent was removed under diminished pressure to a syrupy consistency and 50 ml. of water were added. Addition of a slight excess of dilute ammonia resulted in a voluminous precipitate which was extracted with chloroform. The extract was washed with water and dried. After the chloroform was removed, the crude residue was dissolved in chloroform-benzene (1:10) and chromatographed through 25 gm. of (Brockmann's) alumina. Elution with the same solvent mixture (1:10) yielded at first the dibenzoate. When this was changed to a 1:2 solvent mixture, the monobenzoate followed.

The monobenzoate crystallized as needles from benzene which melted under the microscope at 260–262°. Yields generally averaged about 80 per cent.

$C_{34}H_{47}O_3N$. Calculated, C 78.87, H 9.15; found, C 78.77, H 9.15

The dibenzoate also crystallized as needles from methanol-acetone with a micro melting point of 186–187.5°.

$C_{41}H_{51}O_4N$. Calculated, C 79.19, H 8.27; found, C 79.31, H 8.56

By raising the temperature or increasing the time of heating, the yield of the dibenzoate was increased considerably.

Δ^5-Solanidene-3(β)-ol-12-one Benzoate—A solution of 37 mg. of CrO_3 in 4 ml. of 90 per cent acetic acid was added dropwise to 260 mg. of rubijervine monobenzoate dissolved in 3 ml. of the same solvent and maintained at about 15°. An immediate precipitate formed which redissolved on agitation. After standing 16 hours at room temperature, about two-thirds of the solvent was removed under diminished pressure. 75 ml. of H_2O were then added to the viscous syrup, followed by neutralization with dilute ammonia. The copious precipitate was extracted with chloroform and the extract was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was crystallized from acetone.

It formed long rods which melted at 214–216° and solidified into large slabs or blades which again melted at 233–236°. For analysis it was dried at 110° and 0.2 mm.

$C_{34}H_{45}O_3N$. Calculated, C 79.18, H 8.80; found, C 79.16, H 8.82

In several runs yields of 70 to 75 per cent were obtained.

Δ^5-Solanidene-3(β)-ol-12-one—42 mg. of the above benzoate were dissolved in 0.8 ml. of benzene and refluxed gently with 10 ml. of 1 per cent methanolic KOH solution for 25 minutes. When about two-thirds of the solvent were removed under reduced pressure, needles separated. When twice recrystallized from methanol-ether, it formed long silky needles which melted under the microscope at 236–238°.

$[\alpha]^{28}_D = +45°$ ($c = 0.89$ in chloroform)
$C_{27}H_{41}O_2N$. Calculated, C 78.77, H 10.05; found, C 78.60, H 10.09

The ultraviolet absorption spectrum of the substance shows only carbonyl absorption, as given in Fig. 1.

Semicarbazone of Δ^5-Solanidene-3(β)-ol-12-one Benzoate—180 mg. of the keto benzoate in benzene were concentrated to dryness *in vacuo* to insure removal of any acetone of crystallization and then dissolved in 50 ml. of warm 95 per cent ethanol. A semicarbazide acetate solution was prepared from 0.2 gm. of potassium acetate in 2 ml. of 95 per cent ethanol and 0.2 gm. of semicarbazide hydrochloride in 0.6 ml. of H_2O. The filtrate from precipitated KCl was added to the above solution. The mixture was refluxed for 3 hours and allowed to stand overnight. After removal of about two-thirds of the solvent under reduced pressure, 50 ml. of H_2O were added and the solution was made slightly alkaline with ammonium hydroxide. The resulting copious precipitate was extracted with chloroform and the solution was thoroughly washed with water and dried over Na_2SO_4. The white solid obtained from the extract could not be crystallized. The amorphous residue (220 mg.) was thoroughly digested with cold ether, collected, and dried at 110°. 132 mg. of white amorphous solid were recovered, which decomposed above 265° with preliminary discoloration. In four runs the analyses all indicated low C and N values, even when pyridine was used as the solvent, as recommended by Dutcher and Wintersteiner (10). A satisfactory analysis was obtained only after the material was thoroughly washed with ether with a considerable loss of the substance.

$C_{35}H_{48}O_3N_4$. Calculated, C 73.39, H 8.45, N 9.78
Found, " 73.30, " 8.42, " 9.93

Reduction of Semicarbazone to Solanidine—125 mg. of the above semicarbazone placed in a bomb tube with sodium ethylate solution prepared from 125 mg. of sodium and 5 ml. of absolute alcohol were heated for 8 hours at 200–210°. The contents of the tube were mixed with 100 ml. of H_2O and the opaque solution was acidified with dilute HCl. A practically clear solution resulted which was extracted thoroughly with ether to remove benzoic acid. 20 mg. of the latter were recovered. The aqueous phase was next made alkaline with ammonium hydroxide and the resulting precipitate was extracted with ether. The washed and dried extract, after removal of ether, yielded 60 mg. of white solid, which was dissolved in benzene and chromatographed through 3 gm. of alumina. 20 mg. of substance were eluted with benzene-ether (9:1). When this was recrystallized twice from acetone, needle-shaped crystals were obtained which melted under the microscope at 214–218°. This substance gave no depression in melting point with an authentic specimen of solanidine (micro melting point 215–218°).

$$[\alpha]_D^{20} = -27° \ (c = 0.36 \text{ in chloroform})$$

Prelog and Szpilfogel reported $[\alpha]_D = -27.0° \pm 4°$ (9).

$C_{27}H_{43}ON$. Calculated, C 81.54, H 10.91; found, C 81.51, H 10.85

In another run the crude substance obtained from the Wolff-Kishner reduction was directly crystallized from acetone and was used for the following acetylation.

32 mg. of the base dissolved in 0.8 ml. of pyridine were treated with 0.4 ml. of acetic anhydride and allowed to stand for 17 hours at room temperature. The bulk of the solvent was removed *in vacuo* and 25 ml. of H_2O were added to the residue. The aqueous phase was made slightly alkaline with dilute ammonia and the flocculent mass was extracted with ether. The latter left a residue which, when recrystallized twice from alcohol, formed flat blades which melted at 207–209°.[1] This showed no depression in a mixed melting point with authentic acetylsolanidine.

$C_{29}H_{45}O_2N$. Calculated, C 79.04, H 10.30; found, C 79.20, H 10.32

Dihydrorubijervine Monobenzoate and Dibenzoate—0.2 ml. of benzoyl chloride was added to 0.175 gm. of dihydrorubijervine (2) dissolved in 5 ml. of benzene and 5 ml. of pyridine. The solution was heated in an oil bath at 110–120° for 75 minutes. After partial concentration and addition of water, the solution was made alkaline with dilute ammonia and the precipitated mass was filtered and dried. This material was chromatographed over alumina. The dibenzoate (120 mg.) was obtained by elution with benzene-ligroin (3:1), while the monobenzoate (35 mg.) followed in the benzene-ether (2:1) eluate.

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[1] Schöpf and Herrmann report 206–208° (15).

The dibenzoate crystallized from benzene as needles which melted at 266–269°.

$C_{41}H_{53}O_4N$. Calculated, C 78.93, H 8.56; found, C 79.06, H 8.47

The monobenzoate was crystallized from methanol-acetone. It formed rosettes of needles and melted at 187–190°.

$C_{34}H_{49}O_3N$. Calculated, C 78.57, H 9.50; found, C 78.70, H 9.46

Solanidane-3(β)-ol-12-one Benzoate—30.5 mg. of chromic oxide dissolved in 3.5 ml. of 80 per cent acetic acid were added dropwise to 156 mg. of the above monobenzoate in 2 ml. of acetic acid. The mixture was allowed to stand at room temperature for 80 minutes. After concentration, 125 ml. of H_2O were added, followed by excess dilute ammonia. The voluminous mass was extracted with chloroform. The washed and dried extract yielded, after removal of the solvent, the crude product which was crystallized from a mixture of acetone and benzene. It formed rosettes of needles which melted at 236–241°.

$C_{34}H_{47}O_3N$. Calculated, C 78.87, H 9.15; found, C 78.77, H 9.18

The above compound was treated with selenious acid according to the procedure of Schwenk and Stahl (11), but nothing definite could be isolated from the darkly colored solution.

Solanidane-3,12-dione—0.25 gm. of CrO_3 dissolved in 10 ml. of 80 per cent acetic acid was added dropwise to 0.31 gm. of dihydrorubijervine (2) dissolved in 25 ml. of glacial acetic acid. After 1 hour at 27–29°, the mixture was diluted and extracted with chloroform. The chloroform phase was washed with 5 per cent $NaHCO_3$, then with H_2O, and dried over Na_2SO_4. After removal of the solvent, the crude product was crystallized from acetone. It formed large rhombic crystals which probably contained solvent. When viewed under the microscope, it melted at about 215° and then solidified as small platelets which melted again at 242–244°. It was dried at 110° and 0.2 mm. pressure. The ultraviolet absorption spectrum of the compound is shown in Fig. 1.

$[\alpha]_D^{28} = +119°$ ($c = 1.19$ in chloroform)

$C_{27}H_{41}O_2N$. Calculated, C 78.78, H 10.05; found, C 78.70, H 9.95

When this substance was refluxed in methanolic alkali for 2 hours, it was recovered unchanged.

Disemicarbazone—The procedure of Dutcher and Wintersteiner was closely followed (10). A solution of 500 mg. of the above diketodihydro derivative in benzene was evaporated to dryness *in vacuo* to remove any solvent of crystallization. 500 mg. of semicarbazide hydrochloride dissolved in 1.5 ml. of H_2O were treated with a solution of 500 mg. of potassium acetate in 5 ml. of absolute alcohol. The filtrate from KCl was added to the above diketodihydro derivative dissolved in 10 ml. of pyridine and 8 ml. of absolute alcohol. After the addition of 5 ml. of water, the mixture was gently warmed on the steam bath for a half hour and 3 ml. of chloroform were then added to dissolve the precipitated solid. After standing for 70 hours, the mixture was poured into 200 ml. of H_2O and extracted with chloroform. When the crude chloroform residue was thoroughly washed with ether and dried, 540 mg. of a slightly colored amorphous substance were obtained. It began to discolor at about 260° and charred completely at 300°. When this was directly analyzed without further purification, the nitrogen value was low, a result obtained in four runs. Satisfactory figures resulted only when the material was dissolved in hot alcohol-water and allowed to separate slowly by gradual cooling. About half of the material was lost in this purification.

$C_{29}H_{47}O_2N_7$. Calculated. C 66.25, H 9.01, N 18.65
Found. " 66.10, " 9.16, " 18.30

Wolff-Kishner Reduction of Disemicarbazone—A mixture of 0.25 gm. of sodium dissolved in 7 ml. of absolute ethanol and 280 mg. of the disemicarbazone was heated in a bomb tube for 8 hours at 200° ± 10°. The contents of the tube were poured into 100 ml. of water and extracted with ether. Difficulty was encountered because of emulsification. Subsequent extraction with chloroform proved less troublesome. After removal of the solvents, the material in each case was crystallized from acetone. From the mother liquor of each fraction a small amount of substance which melted at 272–274° was obtained, but it was not investigated further. Since the main fractions were not homogeneous, they were recombined (65 mg.) and chromatographed through 3 gm. of alumina. Elution with benzene yielded in succession two components, a low melting fraction (28 mg.) and a higher melting one (37 mg.). The latter, when recrystallized twice from acetone, formed long needles, which melted at 218–220° and showed no depression when mixed with authentic solanidanol-(3β).

$[\alpha]_D^{30} = +31°$ ($c = 0.70$ in chloroform); $[\alpha]_D = +28.2°$ (±4°) was reported by Prelog and Szpilfogel (9) for solanidanol-(3β).

$C_{27}H_{45}ON$. Calculated, C 81.14, H 11.35; found, C 81.20, H 11.20

The above low melting fraction crystallized as rosettes of needles from 95 per cent ethanol, which melted at 164–166°.[2] This substance is believed to be solanidane. Unfortunately no analysis was obtained with it.

$[\alpha]_D^{23} = +36°$ ($c = 0.43$ in chloroform)

$[\alpha]_D^{17} = +33.1°$ (±2°) was reported by Prelog and Szpilfogel (9).

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[2] Bergel and Wagner give 163–164° (16); Dieterle and Rochelmeyer give 164–165° (17).

All analytical work was performed by Mr. D. Rigakos of this laboratory.

SUMMARY

Rubijervine has been shown to be solanidine with an additional OH group by the conversion of the monoketo derivative of rubijervine into solanidine and by the conversion of the diketodihydro derivative of rubijervine into solanidanol-(3β). Possible positions for the second hydroxyl group have been discussed and the provisional conclusion reached that it is a 12(α)-hydroxyl group.

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INHIBITION OF THE ENZYMIC OXIDATION OF XANTHINE AND XANTHOPTERIN BY PTERIDINES*

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Kalckar *et al.* (1) found that xanthine and xanthopterin oxidases are inhibited by certain preparations of folic acid and that this inhibition is due to the presence of 2-amino-4-hydroxypteridine-6-aldehyde (2). This aldehyde is a fission product of folic acid, from which it is formed by irradiation. In the present work it is shown that several other pteridines exert an inhibitory effect on both enzyme reactions, and studies on the mechanism of this inhibition are described. In the course of these investigations evidence that xanthine and xanthopterin oxidases are identical has accumulated.

Methods

Xanthine and xanthopterin oxidase activities were determined spectrophotometrically according to the method of Kalckar (3). The increase in absorption at 290 mμ (uric acid) and 330 mμ (leucopterin), respectively, was used as a measure of activity. Enzyme and substrate were mixed and subsequently were transferred into a cuvette, and within 1 to 2 minutes the first reading was taken. The total volume of the reaction mixture was 3.0 to 3.2 ml., including 1 ml. of 0.1 M buffer of pH 7.5 or 8.5. Except for the enzyme, the contents of the first cuvette (reference) were the same as those of the second cuvette in which the enzyme reaction was followed. The enzyme concentration was such that a constant reaction rate was obtained for 10 to 20 minutes or longer, depending on the amount of substrate present. The actual amount of xanthine or of xanthopterin oxidized, when necessary, was calculated from the ΔE_{290} and ΔE_{330} values corresponding to the buffer used (see the next section).

EXPERIMENTAL

Preparations—Xanthine oxidase was used, prepared from cream according to the method of Ball (4), except that the last purification step in this procedure was omitted (enzyme I). Another xanthine oxidase preparation was also used; this was prepared by the complete Ball procedure and sub-

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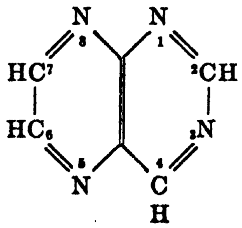
sequent adsorption on an alumina column and elution with sodium pyrophosphate buffer, 0.1 M of pH 8.5 (enzyme II). Xanthopterin oxidase was prepared from whey, according to Kalckar and Klenow (5). The fraction precipitating between 25 and 38 per cent ammonium sulfate saturation was dissolved in sodium pyrophosphate buffer (approximately 0.01 M), pH 8.5, without any further purification (enzyme III). This preparation gave vigorous gas development with H_2O_2, a finding that indicates the presence of catalase. Xanthine (Fisher), after being dissolved in alkali, was precipitated with acid, and the washed product, after recrystallization from boiling water, was dried over sulfuric acid. Xanthopterin (2-amino-4,6-dihydroxypteridine (Lederle)) was recrystallized from boiling water and then was dried over sulfuric acid.

The following compounds were used as inhibitors:[1] 7-OH-pterin (isoxanthopterin), 7-COOH-6-OH-pterin, 7-CH_3-6-OH-pterin, 6-CH_3-7-OH-pterin, 6,7-dimethylpterin, 2,4-diamino-6,7-dihydroxypteridine, and 2,4-diamino-6,7-dimethylpteridine were kindly furnished by Dr. George Hitchings of the Wellcome Research Laboratories. 6-COOH-pterin was supplied by Dr. J. J. Pfiffner of Parke, Davis and Company, 6,7-dihydroxypterin (leucopterin) by Dr. L. D. Wright of Sharp and Dohme, and folvite (folic acid, commercial grade), purified folic acid, 4-aminopteroylglutamic acid ("aminopterin"), and 6-"formyl"-pterin (pterin-6-aldehyde) by the Lederle Laboratories Division or the Calco Chemical Division of the American Cyanamid Company, through the courtesy of Dr. T. H. Jukes. All the compounds were dissolved in a drop of alkali; this was diluted to give a concentration of 2×10^{-4} M and the solution was stored in the refrigerator.

ΔE_{290} *(Xanthine) and* ΔE_{330} *(Xanthopterin)*—Kalckar (3) found that the oxidation to uric acid of 1 γ of xanthine per ml., in a glycylglycine buffer of pH 7.5, causes an increase in absorption ($E_{1\ \text{cm.}}$) of 0.066 at 290 mμ. We found the same value when using a glycylglycine buffer. In a phosphate buffer of pH 7.5, however, this value was 0.056. This difference is due to the fact that uric acid absorbs to a somewhat greater degree in a

[1] To avoid the too frequent use of the cumbersome term, 2-amino-4-hydroxypteridine, this is referred to as "pterin." Throughout this paper the following numbering system of the pteridine ring system has been used:



glycylglycine buffer of pH 7.5 than in a phosphate buffer of the same pH, while the absorption of xanthine is considerably lower. This is caused, apparently, by an interaction with the buffer.

In a pyrophosphate buffer of pH 8.5, we found a ΔE_{290} value for xanthine of 0.047, and in a glycylglycine buffer of pH 8.5 it was approximately 0.054. The $\Delta E_{1\text{ cm.}}$ (330 mμ) for xanthopterin (1 γ per ml.) was estimated as 0.037 in a pyrophosphate buffer of pH 8.5 as well as in a glycylglycine buffer of the same pH, while at pH 7.5, and in both types of buffers used, this value was approximately 0.031, as was found by Kalckar and Klenow (1).

The $\Delta E_{1\text{ cm.}}$ values, expressed on a molar basis, were as follows:

$\Delta E_{1\text{ cm.}}$ per μM per ml.	Phosphate, pH 7.5	Pyrophosphate, pH 8.5	Glycylglycine, pH 7.5	Glycylglycine, pH 8.5
290 mμ (xanthine)	8.51	7.14	10.05	8.21
330 " (xanthopterin)	5.55	6.63	5.55	6.63

Influence of Substrate Concentration and pH on Reaction Rate—Each enzyme preparation showed xanthine oxidase activity as well as xanthopterin oxidase activity. At lower substrate concentrations, however, and depending on the pH, the reaction rate for the oxidation of xanthine was as much as 25 times as high as for xanthopterin. Thus, to obtain a measurable activity for the oxidation of xanthopterin, the enzyme had to be present in a concentration 10 to 20 times that required for xanthine. The reaction rate at room temperature was maximum at a substrate concentration of about 6×10^{-5} M for xanthine as well as for xanthopterin and at both pH values used. Under the experimental conditions, therefore, the enzyme was "saturated" with substrate. At pH 7.5, higher substrate concentrations tend to decrease the reaction rate. This inhibition does not occur at pH 8.5, at concentrations up to 10^{-3} M.

No efforts were made to determine the precise Michaelis constants, since this involves the measurement of reaction rates less than maximum. Such rates cannot be measured very accurately at substrate concentrations of about 10^{-5} M and lower, even with this method. It appears, however, that these constants are of the order of magnitude of 10^{-6}, with the exception of that of xanthine at pH 8.5, which seems to be higher. From the data available it appeared also that the K_m for xanthopterin was smaller than for xanthine at both pH values.

At a substrate concentration of 6.6×10^{-5} M and at pH 8.5, the rate of oxidation of xanthine is approximately 1.6 times the rate at pH 7.5, while in the case of xanthopterin (same concentration) the rate at pH 8.5 was found to be slightly lower than at pH 7.5.

Inhibition of Xanthine Oxidase by Pteridines—This inhibition was measured by determining the activity of a sample of the enzyme as described above and by comparing this with the reaction rate in a third cuvette having the same contents as the second, except that a certain amount of inhibitor was present in the same volume. From these data the percentage inhibition was calculated. Changes or differences in inhibitory power were measured in the fourth cuvette. In the concentrations used, and at the wave-lengths at which the measurements were carried out, the contribution of the inhibitors to the absorption was of no practical consequence.

All simple pteridines tested inhibit xanthine oxidase to some degree. Xanthopterin, for instance, inhibits the oxidation of xanthine, while it can be used as a substrate for the enzyme preparation at the same time. During the time of measurement, however, and with the amount of enzyme used for determining xanthine oxidase activity, the amount of xanthopterin oxidized is almost negligible. Kalckar and Klenow (5) found that the inhibitory power of pterin-6-aldehyde greatly decreased upon previous treatment with a similar enzyme preparation. Presumably the situation for this inhibitor is comparable to that with xanthopterin. The pterin-aldehyde is converted by the enzyme preparation to a substance with a much weaker inhibitory power, and xanthopterin is oxidized to leucopterin, which has a weaker inhibitory effect on xanthine oxidase (Table II). The rate of these conversions is much slower than the rate of oxidation of xanthine with the same amount of enzyme preparation. These considerations led to the treatment of the other inhibitors with an excess of enzyme during a long time interval. This treatment was carried out as follows. A sample of each inhibitor was incubated at 38° for 20 hours with and without the enzyme at pH 8.5 (enzyme III); then the tubes were heated for 5 to 10 minutes at 100°. After cooling to room temperature, approximately one-tenth of the original amount of enzyme was added and the inhibition was measured, as described above, with xanthine as substrate. The results of these experiments are recorded in Table I.

The inhibitory power of a commercial grade of pteroylglutamic (folic) acid (folvite) does not disappear completely following treatment with the enzyme, nor does that of the 6-aldehyde. Highly purified samples of pteroylglutamic acid and of the corresponding 4-amino compound ("aminopterin"), on the other hand, do not show any inhibition after such treatment. Kalckar and coworkers (5) have observed that no change in the absorption spectrum of folic acid occurred during treatment with the enzyme; nor was there any change in the growth-promoting effect on *Lactobacillus casei* and *Streptococcus faecalis* R. For "aminopterin," as well, we found no change in the absorption spectrum. Thus, the disappearance of the inhibition presumably is due only to an enzymic modification of an impurity (the 6-aldehyde), the latter being present in a quantity too small to affect the absorption spectrum. The inhibitory power of the aldehyde is in the order of magnitude of 100 times higher than that of xanthopterin (Table II). Under the conditions given in Table I, before treatment, purified pteroylglutamic acid and "aminopterin" have about the same inhibitory effect as xanthopterin, which indicates that only very little of the aldehyde is present. This amount is apparently too small to give a measurable inhibition after it has been altered by the enzyme treatment. It is unlikely that the remaining inhibition caused by folvite is caused by the altered aldehyde, since in that case it would be necessary to postulate the presence of an unreasonably high percentage of this compound. It is not impossible, therefore, that folvite contains another inhibitory substance in addition to the 6-aldehyde. The decrease in the inhibitory effect of pteroic acid following enzyme treatment also can be accounted for on the basis of the same 6-aldehyde.

TABLE I

Inhibition of Oxidation of Xanthine by Pteridines

Influence of incubation of the inhibitors with the crude enzyme at 38°.

Pteridine	Concentration in reaction mixture	Time of incubation	Per cent inhibition after incubation*	
			Without enzyme	With enzyme
	M × *10*$^{-6}$	*hrs.*		
Folvite	2.3	0	98	
"	2.3	¼		90
"	2.3	½		76
"	2.3	2	96	66
"	2.3	24	95	50
Purified folic acid	2.3	20	71	0
"Aminopterin"	2.3	20	72	0
Pterin-6-aldehyde	3.1	20	100	64
Pteroic acid	3.3	20	100	82
Xanthopterin	3.3	20	72	23
Isoxanthopterin	6.6	20	95	93
7-COOH-xanthopterin	5	20	62	62
6-COOH-isoxanthopterin	7.5	20	23	43†
7-CH_3-xanthopterin	7.7	20	91	89
6-CH_3-isoxanthopterin	7.7	20	89	85
6-COOH-pterin	5.5	20	15	41†
Leucopterin	3.2	20	34	26
2,4-Diamino-6,7-dihydroxypteridine	4	20	76	56

* Substrate concentration 6.3 × 10^{-5} M, pH 8.5.

† Inconsistent.

Compounds which gave an appreciable decrease in inhibition following incubation with the enzyme preparation, but in which the presence of the 6-aldehyde is not so readily postulated, are 2-amino-4-hydroxy-6,7-dihydroxypteridine (leucopterin) and 2,4-diamino-6,7-dihydroxypteridine. Also xanthopterin, after treatment with the enzyme, has a lower inhibitory power than the corresponding amount of leucopterin. 6-Carboxypterin and 6-carboxy-7-hydroxypterin, on the other hand, often showed an increase in inhibitory power. The results with these two compounds were rather inconsistent, but this was not due to extreme lability. Old solutions of the compounds that had been standing for months in the refrigerator, during which time they had been exposed frequently to indirect light for several minutes, showed practically the same inhibitory effect as a freshly prepared solution, when this was determined under exactly the same conditions. As yet, no explanation can be given for the exceptional behavior of the two 6,7-dihydroxy and the two 6-carboxy compounds when treated with the crude enzyme preparation.

TABLE II

Influence of Ratio of Concentration of Substrate to Inhibitor on Inhibition of Oxidation of Xanthine (Xt) and of Xanthopterin (Xtp) by Pteridines*

The results are expressed in per cent.

Inhibitor	Substrate	Molarity inhibitor						
		6.6×10^{-6}	3.3×10^{-6}	1.3×10^{-6}	3.3×10^{-6}	3.3×10^{-7}	10^{-7}	10^{-8}
		Ratio, molarity, substrate to inhibitor						
		1	2	5	10	20	66	660
Xanthopterin	Xt	91	80	59	40			
Isoxanthopterin	Xt	96	91	84	63			
	Xtp		64		16			
Leucopterin	Xt	60	34	19	3			
	Xtp		13		0			
7-CH$_3$-xanthopterin	Xt	92	75	62	50			
	Xtp		24		6			
7-COOH-xanthopterin	Xt	88	64	52				
	Xtp		15					
6,7-Di-CH$_3$-pterin	Xt	58	45	15	9			
	Xtp		3		0			
6-CH$_3$-isoxanthopterin	Xt	81	73	54				
	Xtp		24					
Pterin-6-aldehyde	Xt		100			96	82	48
	Xtp		100			71	46	3

* pH 8.5.

The inhibitory power of the remaining compounds in Table I was practically unchanged on incubation with an excess of the enzyme preparation and is characteristic for each of the pteridines. Under controlled conditions it can be used as a physical constant for identification. Therefore, these compounds were preferred for use in further experiments.

Inhibition of Xanthopterin Oxidase by Pteridines—For experiments on xanthopterin oxidase, the same enzyme preparations were used as for those on xanthine oxidase, but in a 10 to 20 times larger amount. In all other respects measurements of the inhibition were carried out in the same manner as for xanthine oxidase. It was found (Table II) that in general the same compounds which inhibit the oxidation of xanthine also inhibit xanthopterin oxidase, although to a much lower degree at the same pH and the same molar concentration of substrate and inhibitor. Those compounds which showed the weakest inhibition for xanthine oxidase did not manifest any inhibition in this case under certain conditions of pH and substrate concentration. Since it was found (see previous section) that xanthopterin inhibits the oxidation of xanthine, the possibility of an inhibition of xanthopterin oxidase by xanthine was investigated. The increase in absorption at 330 mμ in a cuvette containing 6.6×10^{-5} M xanthopterin was compared with the increase in a cuvette to which xanthine also was added in the same concentration. Because of the larger amount of enzyme used for determining xanthopterin oxidase activity, this amount of xanthine would normally have been oxidized during a fraction of the time of measurement if no xanthopterin were present. Under these circumstances, however, this reaction is inhibited to about 90 per cent. The rate of oxidation of xanthopterin was found to be slightly (not more than 10 per cent) lower than in the control. It was also lower than the rate in a cuvette in which the enzyme had been given time to oxidize the xanthine before xanthopterin was added, indicating that xanthine has a slight inhibitory effect on xanthopterin oxidase, but uric acid has none under the conditions of the experiment (see "Discussion").

Influence of Substrate Concentration and pH on Inhibition—Table II shows that the extent of inhibition is entirely dependent on the substrate concentration. At a definite pH and concentration of the inhibitor, the degree of inhibition decreases as the concentration of the substrate increases. In some cases, when the xanthopterin oxidase activity was measured, no inhibition was observed if, on a molar basis, 10 times as much substrate as inhibitor was present.

The inhibitory power also is strongly influenced by the pH. Isoxanthopterin and most of the other inhibitors inhibit more strongly at pH 8.5 than at pH 7.5, while 7-COOH-xanthopterin inhibits more strongly at the lower pH (see "Discussion"). Some of the data on the influence of the pH on the inhibition (per cent) are given below. These data were obtained with a substrate (xanthine) concentration of 6.6×10^{-5} M, while the inhibitor was present in a concentration of 3.3×10^{-5} M.

Isoxanthopterin		7-COOH-xanthopterin	
pH 7.5	pH 8.5	pH 7.5	pH 8.5
58	91	74	64

Changes in the pH had the same relative effect on the extent of inhibition when xanthopterin was used as the substrate.

Evidence for Identity of Xanthine and Xanthopterin Oxidases—As mentioned above, all enzyme preparations, whether derived from cream or whey and including the most purified (enzyme II), exhibit xanthopterin oxidase as well as xanthine oxidase activity. When the activity of different preparations was determined under the same conditions of pH and substrate concentration, the ratio of xanthine to xanthopterin oxidase activity (expressed as moles oxidized by a certain amount of enzyme per unit time) was practically the same. At low substrate concentrations this ratio tended to be higher in less purified preparations. By using the enzyme in low concentration and increasing the amount of substrate, this ratio could be brought back to approximately that of the more purified preparations. This procedure makes the influence of impurities on the two reactions more comparable, since in the case of xanthopterin 20 times as much enzyme are used as when xanthine is the substrate. The value of the ratio evidently depends on the pH, since the two activities are influenced differently by the pH. For instance, at pH 8.5 and a substrate concentration of 6.4×10^{-5} M, this value is 20 to 25, while, at pH 7.5 and a substrate concentration of 1.6×10^{-3} M, it is only about 10.

Partial heat inactivation of enzyme I or of enzyme III by heating 4 minutes at 60° decreased both activities 30 to 35 per cent.

When enzyme I is treated with H_2O_2 in 10^{-2} M concentration for 15 minutes at room temperature before the dilutions for measuring the activities are made, the activity is decreased in both cases to about 30 per cent.[2]

The pteridines used inhibit xanthopterin oxidase as well as xanthine oxidase activity. At the same pH the sequence of inhibitory power is independent of the substrate used (Table II), although the extent of

[2] No definite conclusions could be drawn from experiments on the inactivation by HCN (6, 7) and by *p*-chloromercuribenzoate, on account of the interaction of these compounds with the substrates. For instance, a solution of xanthopterin formed a yellow color when treated with HCN, and at higher concentrations a reddish precipitate settled. Glutathione "inhibits" the oxidation of xanthopterin, presumably because it reduces this compound to dihydroxanthopterin (8).

inhibition of the oxidation of xanthopterin is always lower than in the case of xanthine (see "Discussion"). Leucopterin is an exception in this sequence, since it has a relatively higher inhibitory effect on the oxidation of xanthopterin. Leucopterin, however, takes part in the enzymic reaction only when xanthopterin is the substrate. Therefore, its inhibition of the oxidation of xanthopterin cannot be compared to its inhibition of the oxidation of xanthine. Although in the control cuvette to which no inhibitor is added as much leucopterin is formed as in the case of the other inhibitors of the oxidation of xanthopterin, no decrease in reaction rate was observed during the time of measurement of uninhibited xanthopterin oxidase activity; this indicates that the amount of leucopterin formed was apparently too small to cause a significant inhibition. This small amount, nevertheless, may exert a measurable influence if leucopterin has been added to the reaction mixture previously, since the inhibition by leucopterin increases rapidly with its concentration (see Table II). Other inhibitors cannot influence the inhibitory effect of leucopterin formed during the reaction, since they do not participate in the equilibrium of leucopterin with the enzyme.

DISCUSSION

The fact that under all experimental conditions the extent of inhibition is influenced by the substrate concentration (Table II) indicates that this inhibition is brought about by competition between the pteridines and the substrate for combination with the active centers on the enzyme molecule. This may be due to similarity in structure of the purine and the pteridine ring system. The degree of inhibition, therefore, would be dependent upon the relative affinity of the substrate and of the inhibitor for the enzyme. On the basis of the evidence for the identity of xanthine and xanthopterin oxidases and in view of the fact that xanthopterin inhibits the oxidation of xanthine to 90 per cent when present in equimolar concentration (Table II), it would follow that 90 per cent of the active centers of the enzyme will be occupied by xanthopterin and 10 per cent by xanthine. Xanthine, therefore, would inhibit the oxidation of xanthopterin to 10 per cent, which is in agreement with the results obtained experimentally. Also, the fact that the inhibitory power of all the inhibitors at both pH values is lower when xanthopterin is used as the substrate than in the case of xanthine merely indicates that xanthopterin has a higher affinity for the enzyme than has xanthine at pH 7.5, as well as at pH 8.5, and, therefore, is more difficult to displace by the inhibitor than is xanthine.

All the pteridines in Table II inhibit the oxidation of xanthine to more than 50 per cent in equimolar concentration and, therefore, have a greater affinity for the enzyme than xanthine. Most of these compounds are not attacked by the enzyme and the ones that are oxidized (xanthopterin and possibly the 6-aldehyde) show a much lower rate of oxidation than xanthine. The affinity for the enzyme, therefore, does not seem to be determined in the first place by groupings in the molecule that can be attacked. The first factor determining such affinity is probably the general shape of the molecule. The only pterins tested which did not inhibit the enzyme, in concentrations at which the other compounds showed strong inhibition, were folic acid and its 4-amino analogue, "aminopterin." The long side chain of these compounds apparently alters the properties of the molecule to such an extent that it no longer "fits" into the particular pattern on the surface of the enzyme molecule.

On the other hand, the affinity for the enzyme at a definite pH is not the first factor determining which substrate will be attacked at the highest rate. Although the affinity of xanthopterin for the enzyme is higher than that of xanthine, still xanthine is oxidized at a rate up to 25 times as high as xanthopterin. Apparently the inherent tendency of xanthine to be oxidized to uric acid is higher than that of xanthopterin to be oxidized to leucopterin, and the enzyme as a catalyst merely increases the rate of a reaction which potentially could take place without the enzyme.

Xanthine has a higher affinity for the enzyme at pH 7.5 than at 8.5; nevertheless it is oxidized at a higher rate at pH 8.5. On the basis of the same reasoning, this would mean that the oxidation of xanthine to uric acid, also non-enzymically, would proceed more readily at pH 8.5 than at pH 7.5.

In cases in which no alteration of the general shape of the molecule exists, differences in inhibitory power of the pteridines must be caused by other factors which influence the affinity for the enzyme.

All pteridines used are amphoteric to a greater or lesser extent (8), but significant differences in their respective pK_a and pK_b values are demonstrable. Unfortunately, very little is known about such values, although it has been established, for instance, that isoxanthopterin is significantly less basic than xanthopterin, while leucopterin is predominantly acid.[3] In view of the fact that the inhibitory power of the pteridines is strongly influenced by the pH, the possibility arises that the affinity for the enzyme and, therefore, the inhibitory power of the compound is determined to a great extent by such pK values. At a certain pH the acid and basic groups of one pteridine will be ionized to a different degree from those of another. By assuming that combination with the enzyme takes place by means of such acid and basic groups in the pteridines combining with basic and

[3] Determination of such values was attempted with some of the compounds of which the quantity available permitted establishing a titration curve. Low solubility, however, especially at the lower pH values, interfered with obtaining accurate results.

acid groups in the protein, it would be clear that the stability of such combination would depend entirely on the relative pK values of the four groups involved.

Significant in this respect may be the finding that, when the 4-OH group in leucopterin is replaced by an amino group, a compound is obtained which has a higher inhibitory power than leucopterin (at pH 8.5). Such substitution would make the acid and basic groups of leucopterin more balanced. On the other hand, when the same substitution is made in the corresponding 6,7-dimethylpterin, the inhibitory power decreases, presumably because almost no acidity is left after such substitution.

This enzyme, with its variety of substrates and inhibitors, seems to be especially suited for studies on interactions of all these compounds with the enzyme as a protein. Relatively small differences in affinity for the enzyme can be measured accurately and such studies might give information as to the configuration of groupings on the protein as well as to the mechanism of the enzyme reaction.

The author is deeply indebted to Dr. A. D. Welch for his constant interest during the course of this investigation.

SUMMARY

1. The inhibition of the enzymic oxidation of xanthine and xanthopterin by several pteridines has been investigated.

2. The results of these experiments together with many other points of evidence indicate that these oxidations are brought about by the same enzyme.

3. The inhibition is caused by competition of the pteridines with the substrate for combining with the enzyme.

4. The presence of a group which can be oxidized by the enzyme is not the first factor determining the affinity of the pteridine for the enzyme.

5. The extent of inhibition is greatly influenced by the pH, and the possibility of a correlation between pK values and inhibitory power has been discussed.

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THERMAL COAGULATION OF SERUM PROTEINS

I. THE EFFECTS OF IODOACETATE, IODOACETAMIDE, AND THIOL COMPOUNDS ON COAGULATION*

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In the course of studies of the effect of heat on the proteins of human serum it was observed that, at pH 7.4, minute amounts of sodium iodoacetate completely prevented thermal coagulation, while traces of mercaptans markedly enhanced the coagulation. The present paper is an analysis of these observations.

The thermal coagulation of a protein, such as egg albumin, has been postulated (1) to consist of two processes, the denaturation reaction between the protein and hot water, followed by the agglutination of the altered protein in particulate form. From x-ray diffraction studies (2) it has been demonstrated that denaturation of proteins involves liberation or generation of peptide chains which aggregate on coagulation into parallel bundles. This over-all physical change is affected by many factors including pH (3, 4) and the concentration (5) and nature (6, 7) of electrolytes in the solution.

A number of substances prevent thermal coagulation of proteins, such as concentrated thiocyanate and iodide (6), germanin (8), certain sugars in concentrated solution (9, 10), thymus nucleate (11), and certain fatty acids and detergents (12, 13). However, the only agents which inhibit coagulation in the low concentration range in which iodoacetate is active are detergents and thymus nucleate, and the inhibitory effect of the latter substance, unlike that of iodoacetate, is abolished by relatively low concentrations of sodium chloride (11).

Because of implications in pathology (14) most of the experiments described in this paper were carried out with normal human serum in order to secure data on this intact system. However, the principal effects were also observed when solutions of crystallized bovine plasma albumin, crystallized egg albumin, and human serum albumin were employed; preliminary experiments with these simpler systems, which bear on the mechanism of the phenomenon, are included.

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EXPERIMENTAL

Method

Unless otherwise stated, results refer to the standard coagulation test wherein serum or protein solution was diluted to a final volume of 1.5 ml. with M/15 phosphate buffer (pH 7.4) and the mixture heated in a Kahn tube (1.2 × 10 cm.) immersed in a bath of vigorously boiling water. Substances whose effect on coagulation were to be tested were brought to pH 7.4 (phenolsulfonephthalein indicator) with 1 N sodium hydroxide or hydrochloric acid and then dissolved in appropriate concentration in the phosphate buffer. The time of coagulation and the physical state at the end of testing were noted. Coagulation was defined as solidification (clot) or as a viscous liquid condition (gel) as contrasted with more fluid states. All solutions were freshly prepared before use and organic chemicals were recrystallized before being tested. pH was determined with a glass electrode on duplicate samples; the values are expressed as obtained at room temperature.

Pooled human serum, filtered to remove cells and coagula, was used. All of the serum pools contained 32.5 to 35.5 mg. of total protein per 0.5 ml. as determined by standard Kjeldahl methods. The serum was "standardized" against iodoacetate; standard serum was defined as that which, in the test described, coagulated in the presence of 3 to 4.5 μM of iodoacetate but remained liquid when 6 μM or more of iodoacetate were present. Substandard serum was never obtained from healthy individuals. Standard serum in the control tubes always coagulated in 10 to 12 minutes.

In certain experiments, crystallized bovine plasma albumin (Armour) was dissolved in 0.15 N sodium chloride and brought to pH 7.4 with a few drops of dilute sodium hydroxide; 0.5 ml. contained 35 mg. of albumin. Human serum albumin, prepared by the method of Pillemer and Hutchinson (15), was similarly employed in concentrations of 44 mg. per 0.5 ml. Crystalline egg albumin (Armour) was dissolved in phosphate buffer (pH 7.4); 0.5 ml. contained 100 mg.

Results

Effects of pH on Thermal Coagulation of Serum

Practically all the experiments reported in this paper were carried out at pH 7.4. When systems of serum diluted 1:3 with buffer solution are heated, opaque firm clots form if the pH is between 6.6 and 8.1. At pH 4.7 to 6.4 a flocculent curdy precipitate is formed without the appearance of a solid clot. At pH 3.4 to 4.1 translucent clots are formed, the clot being less firm in a system whose pH is below 3.8. At pH 1.7 and at pH 10.0 no coagulation occurs.

Inhibition of Coagulation by Halogenated Acetates

At pH 3.4 and 5.4, iodoacetic acid in amounts up to 60 μM per 0.5 ml. of serum had no effect on the thermal coagulation of serum, a translucent clot forming in the former case and a curdy precipitate in the latter. At pH 7.4 the results were quite different in that small amounts of iodoacetate[1] completely prevented coagulation.

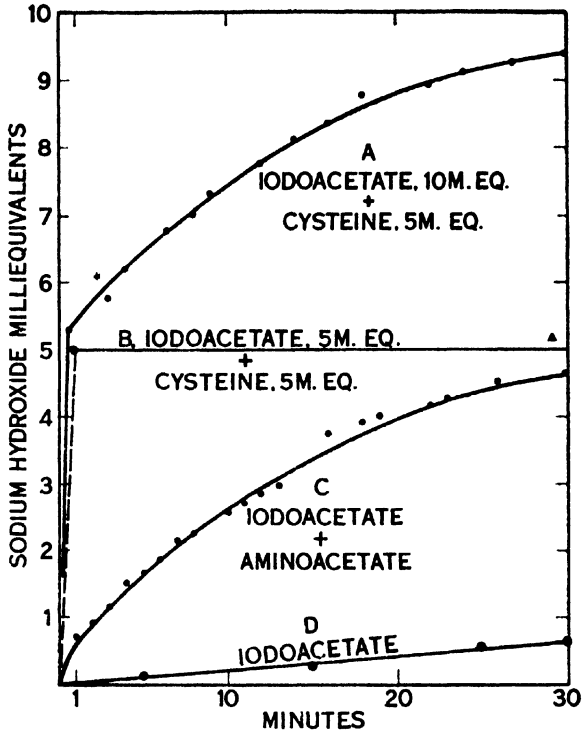

FIG. 1. Rates of reaction of iodoacetate at 98°. The ordinates represent milliequivalents of sodium hydroxide required to keep the reaction at pH 7 to 8. The curves represent the reaction as follows: Curve A, iodoacetate (10 m.eq.) and cysteine (5 m.eq.); Curve B, iodoacetate (5 m.eq.) and cysteine (5 m.eq.); Curve C, iodoacetate and aminoacetate (each 5 m.eq.); Curve D, iodoacetate (10 m.eq.). The curve for the reaction of iodoacetamide (10 m.eq.) with cysteine (5 m.eq.) is practically identical with Curve A. * signifies a negative nitroprusside test for —SH; ▲, a positive test.

In the standard test, coagulation of serum occurred in the presence of 3 μM of iodoacetate but was abolished by 6 to 60 μM (Table I); the latter tubes were boiled for more than 2 hours without solidification or gel formation. With small amounts of iodoacetate (6 to 12 μM) a little turbidity was usually present, while with larger amounts the liquid remained clear. With increasing electrolyte concentrations, increasing amounts of iodoacetate were required to prevent coagulation. For example, when 1500 μM of sodium chloride were added in the coagulation test, complete inhibition required 125 μM of iodoacetate. Potassium chloride and sodium acetate had the same effect quantitatively as sodium chloride.

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[1] A solution of iodoacetic acid neutralized with sodium hydroxide and dissolved in phosphate buffer (pH 7.4) will be referred to throughout this paper as iodoacetate.

The ability of other halogenated acetates to inhibit coagulation was compared under similar conditions. In the standard test, the following amounts of inhibitor were required to prevent coagulation completely: iodoacetate 6 μM, bromoacetate 12 μM, chloroacetate 30 μM. Fluoroacetate did not inhibit heat coagulation of serum.

Table I

Effect of Mercaptans and Iodinated Compounds on Serum Coagulation

0.5 ml. of serum plus reagent in 1.0 ml. of M/15 phosphate buffer (pH 7.4) was heated at 100°.

Compound added	Micromoles of added compound										
	0	0.3	0.6	0.9	1.5	3	6	9	15	30	60
	Time of coagulation										
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
Cysteine	11	10	8.5	6	5	4	2.5	1.5	1		
2,3-Dimercaptopropanol	11	9	9	6	2	1	1	0.5	0.5		
Propyl mercaptan	11						1				
Sodium sulfhydrate	11					4					
Iodoacetate	11	11	19	30	45	90	No clot	No clot	No clot	No clot	No clot
Methyl iodoacetate	10				7		7	3			
Iodoacetamide	10				8	5	3		2	2	
Iodoacetone	10					6	5		3		
α-Iodopropionate	10					12		12		12	8
β-Iodopropionate	10					7	7		24	30	4
Allyl iodide	10				9				9		
Iodoform	10					10			10	10	

Iodoacetate prevents the thermal coagulation of solutions of crystallized proteins in a manner quite similar to its effect on whole serum. When a solution of human serum albumin or crystallized bovine plasma albumin was used, replacing serum in the standard test, dense coagula formed in presence of 1 to 3 μM of iodoacetate; larger amounts completely prevented coagulation and opacity. A 20 per cent solution of crystalline egg albumin coagulated in the presence of 3 to 18 μM of iodoacetate; 30 μM completely prevented coagulation and turbidity formation.

The time required for iodoacetate to react with the serum protein was studied by means of the "quenching" action of an excess amount of cysteine. The reaction between thiol compounds and iodoacetate (Fig. 1) at 100° and pH 7.4 is extremely rapid;[2] so that addition, at any time, of an excess of cysteine blocks any further action of iodoacetate on the protein. A set of tubes containing serum, buffer, and iodoacetate sufficient to prevent clotting completely was heated at 100° and an excess of cysteine added to the different tubes at varying time intervals. The results, given in Table II, show that, at the concentrations employed, the reaction of iodoacetate with serum to prevent coagulation requires about 15 minutes and that, when once this reaction has occurred, cysteine is unable to reverse it.

TABLE II

Rate of Reaction of Iodoacetate with Active Groups in Serum

Tubes containing serum (0.4 ml.) plus iodoacetate (15 μM) in phosphate buffer (0.6 ml.), pH 7.4, were immersed in a bath held at 100°. Cysteine (30 μM) in buffer (0.5 ml.) was added after various intervals and the time of coagulation was recorded.

Time of adding cysteine	Time of clotting after cysteine addition
	min.
Immediately	2
After 5 min.	1.5
" 10 "	4.5
" 15 "	75
" 20 "	No clot

The reaction of iodoacetate with serum proteins can take place to a considerable extent with native proteins at room temperature. Serum was treated with iodoacetate (60 μM per 0.5 ml. of serum) at room temperature, and, after 24 hours, the excess iodoacetate was removed by dialysis. This treated serum showed a greatly decreased ability to coagulate as compared to control serum treated in an analogous manner with sodium acetate. The inhibition was not due merely to adsorbed iodoacetate which dialysis failed to remove, since addition of cysteine in the coagulation test of the iodoacetate-treated serum did not abolish the inhibition.

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[2] At 98° and pH 7 to 8, 0.2 M iodoacetate was found to react completely with the sulfhydryl groups of 0.1 M cysteine in less than 1 minute; the reaction with the amino groups occurred much more slowly, being complete in about 30 minutes (Fig. 1). The reaction of iodoacetamide with cysteine was entirely comparable in rate to that of iodoacetate. The results were obtained by measuring the liberation of acid (HI) and the disappearance of a positive nitroprusside test for sulfhydryl groups.

Although iodoacetate exerts a profound effect on thermal coagulation of serum proteins, it does not prevent precipitation by other means. Standard tests containing 30 μM of iodoacetate were boiled for 30 minutes; all of the solutions were liquid and clear. To the various tubes there were then added 5 ml. respectively of acetone, 22 per cent sodium sulfate, and concentrated nitric acid; a copious precipitate formed in all cases.

Effect of Other Iodinated Compounds

In view of the remarkable inhibition of protein coagulation shown by iodoacetate, a number of other iodinated compounds were investigated; some of these resemble iodoacetate in the reactivity of the iodine atom (Table I). In sharp contrast to iodoacetate, all of these substances were either inert or promoted coagulation, with the exception of β-iodopropionate which inhibited very slightly in a narrow concentration range but promoted coagulation at higher concentrations. In order to give greater opportunity for iodoacetamide to penetrate the protein molecule, 30 μM of this compound were added to the standard test and the mixture was incubated for 24 hours at 37° before being placed in the boiling water bath; the coagulation results were unaltered by this treatment.

Similar differences in the action of iodoacetamide from that of iodoacetate were observed with solutions of crystallized bovine plasma albumin. Iodoacetate completely blocked coagulation, while iodoacetamide markedly enhanced the formation of a firm clot. In the presence of either agent, the opacity usually accompanying the heating of albumin solutions at pH 7.4 was absent; the tube with iodoacetate contained a clear liquid, while the tube with iodoacetamide contained a clear firm clot. Addition of cysteine to the control tube noticeably increased the turbidity of that clot. These results suggest that, although iodoacetate and iodoacetamide differ in their effect on clot formation, they react similarly to prevent turbidity formation.

Differences in the effect of iodoacetate and iodoacetamide on the thermal coagulation of proteins, as well as the involvement of sulfhydryl groups in the formation of opacity, are shown by an experiment in which portions of crystalline bovine plasma albumin were first treated at room temperature with iodoacetate or iodoacetamide. The excess reagent was then removed by dialysis and the behavior of these treated proteins was studied in the coagulation test (Table III). Albumin previously treated with iodoacetate formed neither a clot nor turbidity on heating. Albumin treated with iodoacetamide coagulated rapidly on heating but formed no turbidity. The addition of iodoacetate in the coagulation test of the iodoacetamide-treated protein caused some retardation of coagulation but did not abolish coagulation as it did in the controls. Addition of cysteine to the coagulation tests of either the iodoacetate- or iodoacetamide-treated proteins resulted in opacity of the system.

Enhancement of Thermal Coagulation by Thiol Compounds

Substances containing sulfhydryl groups accelerate the coagulation of serum and protein solutions. Increasing amounts of cysteine up to 15 μM per standard test markedly decreased the time of coagulation, while 2,3-dimercaptopropanol was even more effective (Table I). In addition to hastening the coagulation of serum, sulfhydryl compounds caused coagulation in concentrations of serum so dilute that clotting was not otherwise detectable. Cysteine (30 μM) caused serum to clot when the final serum concentration was 10 per cent; in control tubes with sodium chloride (30 μM) replacing cysteine, coagulation did not occur in concentrations of serum weaker than 20 per cent.

TABLE III

Coagulation of Iodoacetate and Iodoacetamide-Treated Albumin

250 mg. portions of crystallized bovine plasma albumin in 5 ml. of phosphate buffer (pH 7.4) were treated with either 1000 μM of sodium acetate or iodoacetate, or iodoacetamide. After standing at 4° for 48 hours the reaction mixtures were dialyzed against distilled water (3 days at 4°) and lyophylized. Solutions (in 0.15 N saline) of treated protein containing 35 mg. per 0.5 ml. were studied in the standard coagulation test.

Protein used	Added reagent in coagulation test (6 μM)	Time for coagulation	Appearance after heating 15 min.
		min.	
Control (acetate-treated)	None	4	Opaque solid
	Iodoacetate	No clot in 15	Clear liquid
	Iodoacetamide	2	" solid
	Cysteine	2	Very opaque solid
Iodoacetate-treated	None	No clot in 15	Clear liquid
	Iodoacetamide	" " " 15	" "
	Cysteine	" " " 15	Cloudy "
Iodoacetamide-treated	None	3	Clear solid
	Iodoacetate	11	" "
	Cysteine	5	Opaque solid

The inhibition of thermal coagulation of serum by alkyl sulfonates can be neutralized with cysteine. Thus, in the standard test, the inhibitory effect of 6 μM of sodium dodecyl sulfate was overcome by 1.5 μM of cysteine, of 12 μM by 27 μM of cysteine, and of 18 μM by 72 μM of cysteine. 24 μM of sodium dodecyl sulfate inhibited coagulation regardless of the quantity of added cysteine.

Effects of Other Reagents on Thermal Coagulation of Serum

The effects of a variety of compounds on the thermal coagulation of serum were investigated. The ability to promote coagulation in low concentration is not a unique property of sulfhydryl compounds but was observed with salts of heavy metals (mercuric chloride, *p*-chloromercuribenzoate, cuprous chloride, cadmium chloride) and iodoacetamide, compounds which are generally accepted as being able to combine with sulfhydryl groups in proteins (16). Reducing agents (hydroquinone, sodium ascorbate, sodium sulfite, and sodium hydrosulfite) and oxidizing agents (potassium iodate, potassium ferricyanide, potassium persulfate, potassium permanganate, and cumene hydroperoxide) in general promoted coagulation but were somewhat less active than sulfhydryl compounds or heavy metal salts. Salts with no oxidizing or reducing properties were essentially inert in amounts up to 60 μM per standard test; at higher concentrations these electrolytes all promoted coagulation. Alanine, methionine, glucose, and urea were practically inert in low concentrations, although large amounts (1500 μM) of the last two compounds inhibited coagulation.

N-Bromosuccinimide, *N*-chlorosuccinimide, and sodium dodecyl sulfate strongly inhibit coagulation when present in amounts of 15 μM or more, although the former compounds strongly enhance coagulation when present in very low concentration (1.5 to 3 μM).

DISCUSSION

Iodoacetate and iodoacetamide differ greatly in their effect on the coagulation of serum proteins by heat at pH 7.4; the former compound strongly inhibits coagulation, while the latter enhances it. However, both compounds react with serum proteins at room temperature and at 100° and both inhibit the formation of turbidity during the coagulation test. Groups in proteins which are known to react with both iodoacetate and iodoacetamide are amino and sulfhydryl groups (17–23); essentially no differences were observed in the rates of reaction of iodoacetate and iodoacetamide with the model compound cysteine at 98° and pH 7.4 (Fig. 1); at room temperature iodoacetamide is reported to react slightly faster than iodoacetate (21).

$$\mathrm{RSH} + \begin{cases} \mathrm{ICH_2COOH} \rightarrow \mathrm{HI} + \mathrm{RSCH_2COOH} \\ \mathrm{ICH_2CONH_2} \rightarrow \mathrm{HI} + \mathrm{RSCH_2CONH_2} \end{cases}$$

$$\mathrm{RNH_2} + \begin{cases} \mathrm{ICH_2COOH} \rightarrow \mathrm{HI} + \mathrm{RNHCH_2COOH} \rightarrow \mathrm{RN(CH_2COOH)_2} \\ \mathrm{ICH_2CONH_2} \rightarrow \mathrm{HI} + \mathrm{RNHCH_2CONH_2} \rightarrow \mathrm{RN(CH_2CONH_2)_2} \end{cases}$$

The fact that albumin previously treated with iodoacetamide is no longer prevented from coagulating by the usual concentrations of iodoacetate further indicates that both compounds react with the same groups in the protein. Therefore the difference in their effects seems to be associated with the carboxyl end of the iodoacetic acid molecule.

The modified protein, formed by the reaction of iodoacetate with its amino and sulfhydryl groups, would contain a number of additional carboxyl groups which at pH 7.4 would exist chiefly in the forms of anions. The result is an increase in the net negative charge, an effect not unlike that caused by an increase in pH of the system; it is known that at high pH values heat denaturation of albumin does not lead to coagulation. In all probability the mutually repulsive forces of the negatively charged groups prevent close association and agglutination of the denatured protein. Iodoacetamide, although it can react with amino and sulfhydryl groups in the protein, does not introduce new centers of negative charge and therefore does not inhibit coagulation.

There are other instances known in which iodoacetate and iodoacetamide differ in their action on proteins. The fermentation of glucose by yeast extracts is inhibited much more by iodoacetate than by iodoacetamide (21); yet the inhibitory effects of these substances on crystalline urease is in the opposite order (24, 25). The explanation of these effects has been merely speculative and must remain so until the reaction products have been isolated.

The activity of the various halogenated acetates in blocking coagulation is in line with the reactivity of the halogen atom in the compound, as is shown by the relative reaction rates with thioglycolic acid. In these reactions at 20°, Hellström (26) found that the relative velocity constants of iodoacetate, bromoacetate, and chloroacetate were 300, 120, and 1 respectively.

The rôle of sulfhydryl groups in the thermal coagulation of serum proteins (at pH 7.4) is at present obscure, although there seems to be a definite correlation between the presence of sulfhydryl groups and turbidity formation. The marked enhancement of clot formation by added sulfhydryl compounds is not specific but is shown by compounds capable of binding sulfhydryl groups, such as heavy metal salts and iodoacetamide, and also by certain oxidizing and reducing agents.

SUMMARY

1. At pH 7.4, low concentrations of iodoacetate, bromoacetate, and chloroacetate ions, in order of decreasing effectiveness, inhibit thermal coagulation of human serum, crystallized bovine plasma albumin, and egg albumin irreversibly. At pH 3.5 iodoacetic acid does not block coagulation.

2. Substances closely related to iodoacetate, such as methyl iodoacetate, iodoacetamide, and iodoacetone, accelerate rather than inhibit coagulation.

However, both iodoacetate and iodoacetamide inhibit turbidity formation in heated serum and protein solutions.

3. The inhibitory effect of iodoacetate on thermal coagulation at pH 7.4 is postulated as being due to the introduction of centers of negative charge (carboxyl groups) by reaction with the sulfhydryl and amino groups of the protein molecule.

4. Low concentrations of thiol compounds, heavy metal salts, and certain oxidizing and reducing agents markedly accelerate thermal coagulation of serum proteins.

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THE MOLECULAR SIZE AND SHAPE OF THE PANCREATIC PROTEASES

I. SEDIMENTATION STUDIES ON CHYMOTRYPSINOGEN AND ON α- AND γ-CHYMOTRYPSIN

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Although trypsin, α-, β-, and γ-chymotrypsin, and their inactive precursors were obtained in crystalline form by Kunitz and Northrop (1) and by Kunitz (2) more than 10 years ago, detailed molecular-kinetic characterization of these substances is lacking. The present experiments have been undertaken as part of a comprehensive investigation of the proteolytic enzymes of the pancreas which will include a physicochemical study of the reversible denaturation of some of these proteins. This paper constitutes the first part of a project which will include characterization of these proteins by means of sedimentation, diffusion, and viscometric measurements.

EXPERIMENTAL

Proteins—The materials used for these analyses were prepared according to the procedures of Kunitz and Northrop (1) and of Kunitz (2).

Two samples of chymotrypsinogen were used. One of these (ChTg V) was recrystallized eight times from ammonium sulfate and was then dialyzed free of salt against 0.001 N HCl. This solution was concentrated by pervaporation to a concentration of about 8 per cent, and one portion was used as a stock solution for most of these measurements, while the remainder was lyophilized. As reported by Jacobsen (3), the stock solution was found to be stable in the cold for several weeks. In the last portion of this solution used for these measurements, well formed crystals appeared when the solution was warmed to room temperature. The second preparation of chymotrypsinogen (ChTg IV) was recrystallized six times from ammonium sulfate and was then crystallized twice from magnesium sulfate. These crystals were dried to a powder under the coils of a mechanical refrigerator.

Two preparations of once recrystallized α-chymotrypsin were studied. Of these one (ChT I) was dialyzed free of salt against 0.001 N HCl and was lyophilized, whereas the other (ChT IV) was washed free of ammonium sulfate with saturated magnesium sulfate in 0.01 N sulfuric acid and was dried under the coils of a refrigerator.

One sample of γ-chymotrypsin was prepared from the filtrates of two α-chymotrypsin preparations. Part of this preparation was dialyzed free of salt and was lyophilized for these determinations.

The activity of ChT IV and of the preparation of γ-chymotrypsin has been reported in other publications (4–6).

Buffers—Sedimentation constants were determined in 0.18 M NaCl solutions containing buffer salts sufficient to bring the ionic strength to 0.2. Acetate buffers were used for pH 3.86 and 4.99 and phosphate for pH 6.20. One run was made in acetate buffer of pH 4.98 which contained 0.02 M sodium acetate and 0.48 M NaCl. The pH of the buffers was determined with a Beckman model G pH meter and was found to remain essentially constant at all protein concentrations studied.

Methods—The sedimentation analyses were performed with the electrically driven model E ultracentrifuge built by the Specialized Instruments Corporation. The rotor, supplied by the same source, was of the type described by Bauer and Pickels (7) except that the chuck was replaced by a threaded coupling and the lower rotor stem was eliminated.

Most runs were made at a speed of 59,780 R.P.M. The rotation rate was checked frequently by means of a reduced speed counter attached to the drive and in all cases the mean speed over a 30 minute period was within 50 R.P.M. of the indicated speed. This error is probably the limit of error in reading the counter. Speed variations over a shorter interval were of the order of ±100 R.P.M.

The rotor temperature was measured at the beginning and end of each run with a contact thermocouple. The temperature rise per hour at 59,780 R.P.M. was less than 0.6°. The pressure in the vacuum chamber was of the order of 0.2 μ for each run. Although most runs were made at room temperature, the vacuum chamber was refrigerated during acceleration and for about two-thirds of the duration of each run. Refrigeration was discontinued after this interval to avoid undue cooling of the rotor when air was readmitted to the vacuum chamber.

Boundary positions were recorded by means of the Philpot-Svensson optical system (8) with the automatic camera supplied with the instrument. The exposure interval was 8, 16, or 32 minutes. The light source in this instrument is a water-cooled type A-H6 mercury arc. Eastman spectroscopic plates, type I-D, were used with a Kodak No. 16 Wratten filter.

Since the boundaries obtained with all of these materials were highly symmetrical (Fig. 3), boundary displacements were measured to the maximum ordinate of the Philpot-Svensson photograph. Values of s'_{20} were determined by the method of Oncley (9).[1]

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[1] The symbols used throughout this paper are those standardized by Svedberg and Pedersen (7).

The densities and relative viscosities of appropriate dilutions with 0.001 N HCl of the buffers used with the stock solution of chymotrypsinogen were determined experimentally and were found to give good agreement with interpolations of the data presented by Svedberg and Pedersen (7). Other viscosity and density data were taken directly from this reference.

Chymotrypsinogen concentrations were determined by the semimicro-Kjeldahl method. α-Chymotrypsin and γ-chymotrypsin concentrations were determined photometrically at the wave-length of maximum ultraviolet absorption by the method of Kunitz (10). The reference data for this method were supplied by semimicro-Kjeldahl nitrogen determinations.

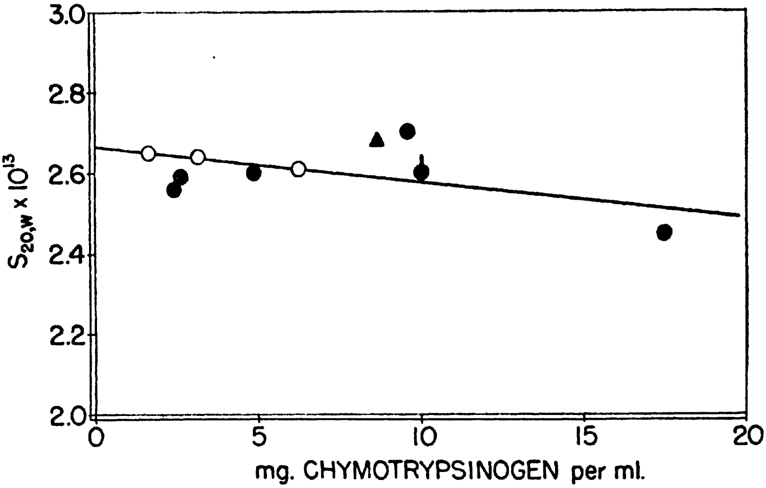

FIG. 1. A plot showing the variation of the sedimentation constant of chymotrypsinogen with protein concentration. Except where indicated, ChTg V was used for these measurements. ●, pH 3.86, ionic strength 0.2. The symbol with the bar at the top indicates the measurement made with ChTg IV. ○, pH 6.20, ionic strength 0.2. ▲, pH 4.99, ionic strength 0.5.

The nitrogen content of chymotrypsinogen was taken as 15.8 per cent (1, 3), while the values of Brand (11) were used for the two chymotrypsins.

The partial specific volume of chymotrypsinogen was found to be 0.73, and this value was assumed for the other two proteins studied.[2]

Results

Chymotrypsinogen—The results obtained with chymotrypsinogen are shown in Fig. 1. Although the scatter of the points on this plot appears to be considerable, it will be noted that the maximum deviation from the least square line is of the order of 4 per cent. The small negative slope of the least square line agrees with the observation that the specific viscosity increment of chymotrypsinogen is of low order, *i.e.* 0.030.[2] Application of this viscosity correction to the least square plot yields a horizontal line.

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[2] Details of this determination together with similar measurements for the other materials will be published in the future.

Although Oncley (9) quotes 3.1 Svedberg units as the value of $s_{20,w}$ for chymotrypsinogen from preliminary results of Hess and Williams, the conditions under which these determinations were made are not stated. The data presented here indicate that $s_{20,w}$ for chymotrypsinogen extrapolated to zero concentration is 2.7 (2.6_6).

Since the molecular weight determinations of Kunitz and Northrop by osmotic pressure measurements (1) were carried out in concentrated salt solutions, one run was made in a buffer whose ionic strength was 0.5. Within the limits of error of the measurement, no change in sedimentation rate was introduced by the higher salt concentration. It will be noted from Fig. 1 that no difference in sedimentation rate was found for the two samples of chymotrypsinogen studied.

Upon completion of the sedimentation, density, and viscosity determinations with chymotrypsinogen, the solutions used were pooled and the protein precipitated by adding solid ammonium sulfate to 0.7 saturation. When this precipitate was treated by the usual crytallization procedure, a heavy crop of well formed chymotrypsinogen crystals was obtained.

α-Chymotrypsin—The data obtained with α-chymotrypsin are shown in Fig. 2. These results seem to resemble qualitatively those reported by various investigators in the Upsala laboratories for human, horse, and cat hemoglobin (7) for which sedimentation constants were found to decrease on dilution. Since the boundaries in all of these determinations were symmetrical (*cf.* Fig. 3) and showed no evidence of a second component, the tentative conclusion is drawn that the α-chymotrypsin monomer, with a sedimentation rate not very different from that of chymotrypsinogen, is in equilibrium with a dimer. The equilibrium between monomer and dimer is shifted toward the dimer by increasing the concentration of α-chymotrypsin or by decreasing the pH. It will be noted from Fig. 2 that a check determination made with ChT IV gave an identical rate of sedimentation with that found for ChT I.

Since the curves shown in Fig. 2 for different pH values merge with increasing α-chymotrypsin concentration into a single line of constant slope, the assumption that points lying on this line represent the sedimentation rate of the pure dimer seems not unreasonable. From a least square extrapolation, with the nine points which are closest to the line indicated, the value of $s_{20,w}$ at zero concentration for the postulated α-chymotrypsin dimer is 3.5 (3.5_2) Svedberg units. Extrapolation of the data at various pH values to the value of $s_{20,w}$ for the monomer was not attempted, since the lines describing the variation of sedimentation rate with concentration are curvilinear. At present the most promising approach to the problem of the molecular size and shape of the monomer seems to be to evaluate the size and shape of the dimer, and, the limits within which the dimensions of the monomer must fall being known from the experimental evidence, to estimate the range of sizes and shapes which will form a dimer of known size and shape.

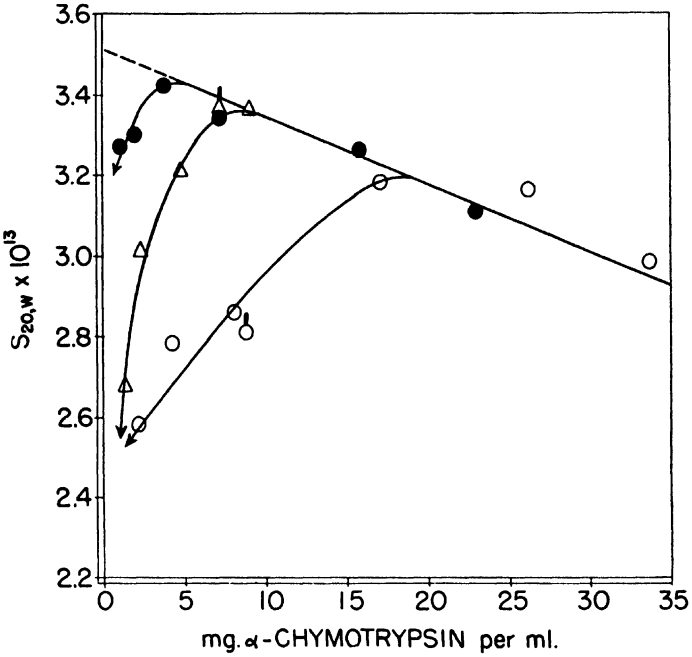

FIG. 2. A plot of sedimentation constant against α-chymotrypsin concentration for three pH values. Except as noted, determinations were made at about 25° with ChT I. ●, pH 3.86, ionic strength 0.2. △, pH 4.99, ionic strength 0.2. The symbol with the bar at the top designates the measurement made with ChT IV. ○, pH 6.20, ionic strength 0.2. The symbol with the bar is for the determination made at 10°.

Since the concentrations of α-chymotrypsin monomer and dimer are postulated to depend upon the total protein concentration, the "tail" of the boundary on the solvent side must represent a higher ratio of monomer to dimer than is present in the bulk of the solution. This portion of the boundary is also in a weaker field and will, therefore, lag slightly. From one edge of the boundary to the other the ratio of monomer concentration to dimer concentration will change progressively until the concentration and composition of the bulk of the solution are attained. The edge of the boundary on the solution side will have the highest concentration of dimer and, because of this fact and the greater field strength, will spread toward the bottom of the cell.[3] Since the boundary of a solution of pure monomer or pure dimer would be acted upon only by diffusion and by the change in field across the boundary, but would not have the superimposed composi-

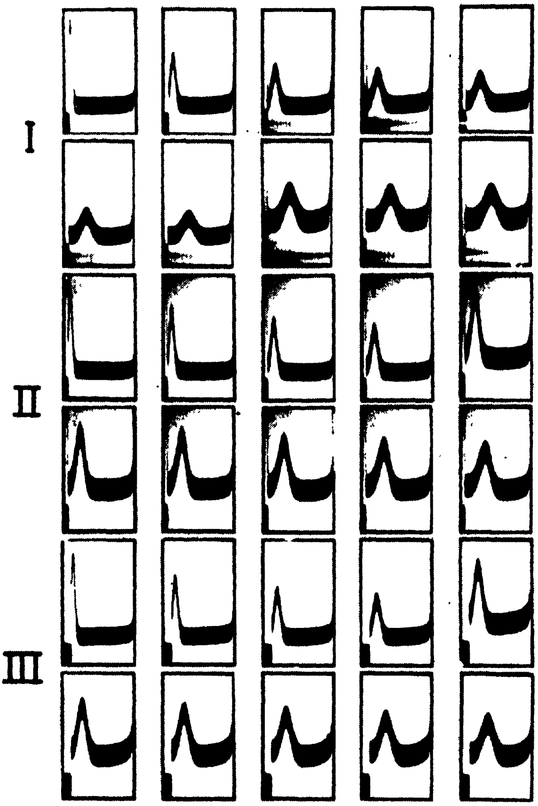

FIG. 3. Typical Philpot-Svensson photographs obtained during sedimentation analyses. In all cases the protein concentration was about 0.9 per cent and the rotation rate was 59,780 R.P.M. Reference lines have been trimmed from these pictures. *I*, chymotrypsinogen; the first seven exposures were made with a bar angle of 60° and an exposure interval of 16 minutes; the last three exposures with a bar angle of 45° at 8 minute intervals; pH 3.86, ionic strength 0.2. *II*, α-chymotrypsin; exposures made at 8 minute intervals; the first four exposures with a 60° bar angle and the remaining with a 45° bar angle; pH 4.99, ionic strength 0.2. *III*, γ-chymotrypsin; 8 minute exposure interval; bar angle 60° for the first four exposures and 45° for the remaining exposures; pH 6.20, ionic strength 0.2.

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[3] The boundary spreading into the solution phase will be somewhat restricted by the greater viscosity of the solution. Close examination of the Philpot-Svensson photographs indicates that boundary spreading is actually less on the solution side of the boundary than on the solvent side.

tion gradient across the boundary, it appears probable that boundary spreading for a solution of intermediate composition will be greater than that for solutions of either pure component. Consequently, as the ratio of monomer to dimer is altered by changing the total protein concentration, a maximum in the apparent diffusion constant should be observed.

The apparent diffusion constants were calculated from the Philpot-Svensson photographs of several of the runs made at pH 6.20 by means of Method III described by Pedersen (7). The results are shown in Fig. 4. Although the accuracy of the points for the two lowest concentrations is low, this result does seem to lend weight to the interpretation given here

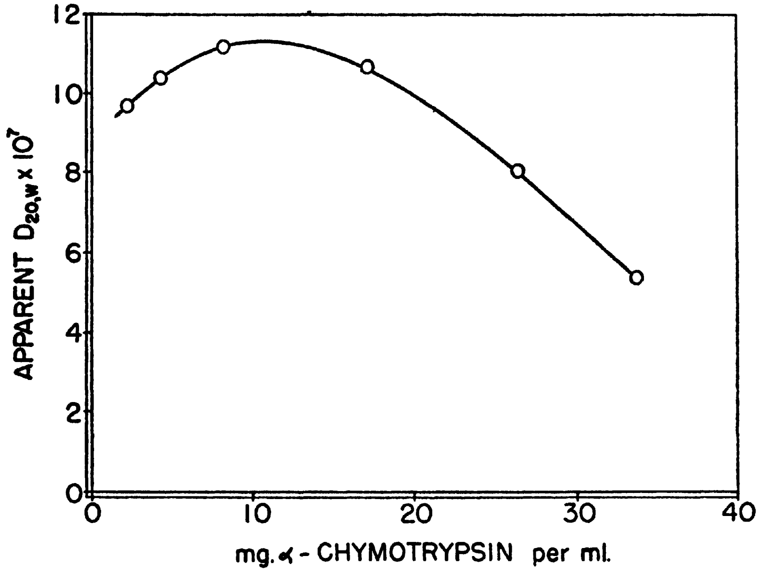

FIG. 4. A plot of apparent diffusion constants, calculated from sedimentation diagrams obtained at pH 6.20 in a buffer of 0.2 ionic strength, against α-chymotrypsin concentration.

The argument that α-chymotrypsin did not undergo any changes during the measurements reported here is based upon the following evidence: (1) The plot of log x against time showed no significant deviation from linearity for any run. (2) When the sedimentation rate was determined at 10° instead of at room temperature, an identical value was found within the limits of error of the determination (*cf*. Fig. 2). Since the viscosity of water is about 50 per cent greater at 10° than at 25°, while an enzymatic reaction would only be expected to proceed at about one-third the rate at 10° as at 25°, these factors are not entirely compensatory. (3) The fact that the sedimentation rates at high concentrations are identical for all pH values indicates that autolysis does not occur at the higher pH values.

(4) The absence of a second peak, or of appreciable asymmetry, indicates that modified protein was not formed.

γ-Chymotrypsin—The results shown in Fig. 5 for γ-chymotrypsin are similar to those obtained with α-chymotrypsin except that a much greater dependence of a postulated dissociation constant of the dimer upon pH is apparent. γ-Chymotrypsin, as would be expected from the molecular weight given by Kunitz (2), sediments at a considerably lower rate than does α-chymotrypsin. Although the extrapolation may be modified by a complete knowledge of the intrinsic viscosities of the components of this system, 3.2 Svedberg units may be tentatively taken as the value of $s_{20,w}$ for the γ-chymotrypsin dimer.

With γ-chymotrypsin, as with α-chymotrypsin, the plots of log x against time were linear for each run and the boundaries were symmetrical for all determinations. Additional evidence that autolysis did not occur at higher pH values is provided by the fact that sedimentation rates increased rather than decreased with increasing pH values.

DISCUSSION

Evidence has been presented that α- and γ-chymotrypsin behave as reversible monomer-dimer equilbrium systems whose dissociation constants increase with pH. The shape of the curves presented in Figs. 2 and 5 can be accounted for by the interaction of the following effects: (1) As the protein concentration is increased, more dimer is formed, so that the sedimentation rate tends to increase. (2) As the total protein concentration increases, the viscosity of the solution increases and the sedimentation rate tends to decrease. Probably the contribution of the dimer to the viscosity of the solution is somewhat greater than that of the monomer.

Attempts at a complete analysis of this phenomenon will be deferred until data regarding the intrinsic viscosity and diffusion constants of the components of the monomer-dimer equilibrium system have been evaluated. However, tentative conclusions may be drawn as follows:

If it is assumed that the monomer of α-chymotrypsin consists of a sphere having a hydration of 0.15 gm. of water per gm. of protein, and that the dimer consists of two spheres of unchanged hydration in contact at one point, the value of f/f_0 for the dimer is 1.10. With this value, the molecular weight of the dimer is 35,000. Conversely, assuming the molecular weight of the monomer to be 17,500 and f/f_0 to be 1.05, the sedimentation rate at zero concentration is 2.34 Svedberg units, a value fitting well the data shown in Fig. 2.

If similar assumptions and calculations are made for γ-chymotrypsin, the molecular weight of the dimer is 31,000 and the sedimentation constant of the monomer is 2.1 Svedberg units, a value somewhat lower than appears probable from Fig. 5 but, in view of the elementary assumptions made, sufficiently near to the observed values to support this interpretation.

From the observed tendency of this equilibrium system to shift toward the monomer as the pH is raised, it may be postulated that the monomer is the active enzyme. Since pH 6 represents the approximate lower limit of activity of this enzyme, studies at higher pH values were not undertaken because of the well known tendency of the active proteases to undergo autolysis in the pH range in which they are enzymatically active (1).

Chymotrypsinogen has not been found to show the phenomenon of dimerization at any pH value or salt concentration studied. From viscosity data for chymotrypsinogen, $f/f_0 = 1.05$. From this value and the sedimentation data presented here, the molecular weight is 22,000. However, with the value for the diffusion constant given by Oncley from preliminary measurements of Hess and Williams (9) (7.9×10^{-7}) and with the sedimentation constant presented here, the molecular weight of chymotrypsinogen is 30,000. This discrepancy between viscometric and diffusion values has been previously noted by Kunitz and Northrop (1). It is anticipated that further studies will clarify this point.

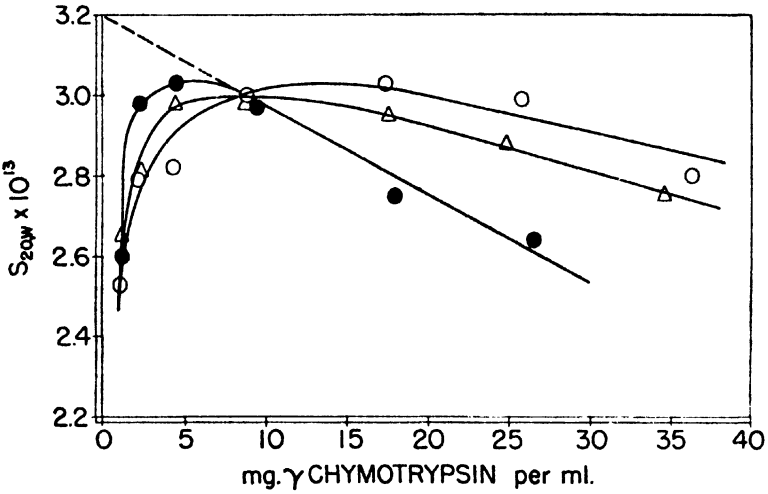

FIG. 5. The variation of the sedimentation constant of γ-chymotrypsin with protein concentration for three pH values. ●, pH 3.86, ionic strength 0.2. △, pH 4.99, ionic strength 0.2. ○, pH 6.20, ionic strength 0.2.

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SUMMARY

The sedimentation constant of chymotrypsinogen at zero protein concentration has been found to be 2.7 Svedberg units over the pH range 3.86 to 6.20 and at ionic strengths between 0.2 and 0.5.

Both α- and γ-chymotrypsins appear to associate to a dimer in solution. The degree of association is increased with increasing protein concentration and is decreased with increasing pH. $s_{20,\,w}$ for the dimer of α-chymotrypsin is 3.5 Svedberg units extrapolated to zero protein concentration. The value of $s_{20,\,w}$ extrapolated to zero protein concentration for the dimer of γ-chymotrypsin is 3.2 Svedberg units. These values have been discussed in relation to the probable molecular weights of these substances.

Addendum—Recently, Dr. John F. Taylor at Washington University, St. Louis, and Dr. Elliot Volkin at the Oak Ridge National Laboratory have made independent determinations of the sedimentation constant of ChTg V at the 1 per cent concentration level. Dr. Taylor used an acetate buffer, pH 5, ionic strength 0.2, and found $s_{20,\,w}$ to be 2.54 and 2.42 from determinations made at 18.4° and at 6.7°, respectively. Dr. Volkin made a determination at a mean temperature of 30.2°, with the phosphate buffer of pH 6.20 used in this laboratory, and found $s_{20,\,w}$ to be 2.43. These determinations were made with Specialized Instruments Corporation ultracentrifuges.

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THE KINETICS OF THE AMIDASE AND ESTERASE ACTIVITIES OF TRYPSIN

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In previous reports from this laboratory investigations of the specific esterase activity of trypsin (1) and of the detailed kinetics of the hydrolysis of specific substrates by chymotrypsin and by carboxypeptidase have been reported (2, 3). In the present paper a reinvestigation of the kinetics of the action of trypsin on benzoyl-L-argininamide and the results of kinetic studies of the action of trypsin on a series of esters of benzoylarginine are presented.

EXPERIMENTAL

Enzyme—The trypsin used for this study (T_V) was prepared according to the procedures of Kunitz and Northrop (4) and of McDonald and Kunitz (5). The enzyme was recrystallized one time.

Substrates and Inhibitor—The preparation of α-benzoyl-L-arginine has been described (1). α-Benzoyl-L-argininamide (BAA) and the methyl, ethyl, and isopropyl esters of α-benzoyl-L-arginine (BAME, BAEE, and BAIPE) were prepared according to the method of Bergmann, Fruton, and Pollok (6). The procedure of these authors was modified for the preparation of the cyclohexyl and benzyl esters of α-benzoyl-L-arginine (BACHE and BABE) in that the esterification was carried out only one time and the reaction mixture was heated on a boiling water bath for 3 hours. The reaction mixtures in all cases were concentrated under reduced pressure and ether was added to precipitate the esters as oils. BAEE, BAIPE, BACHE, and BABE crystallized on standing under ether in a cold box at −20°. These esters were recrystallized by dissolving them in the corresponding alcohol and adding ether to turbidity. As previously reported (1, 6), BAME could not be crystallized.

The analytical results for these materials are shown in Table I.

Methods—The methods for determining the amidase and esterase activity of trypsin have been described (1). The only modification in these procedures was the use of a small mechanical stirrer in place of hand stirring or stirring by nitrogen bubbles in the determination of esterase activity.

Enzyme concentrations were determined by the semimicro-Kjeldahl method.

Results

Action of Trypsin on Benzoylargininamide—Hofmann and Bergmann (7), Butler (8), and Schwert, Neurath, Kaufman, and Snoke (1) have reported that the hydrolysis of benzoylargininamide by trypsin follows first order kinetics. This result is surprising, since it has been generally found, in agreement with the concept established by Michaelis and Menten (9), that adherence to first order kinetics in enzyme systems is apparent rather than real. In recent studies from this laboratory it has been pointed out that the kinetics of enzymatic reactions can be generally treated according to the integrated Michaelis-Menten equation (2, 3).

Accordingly, the hydrolysis of benzoylargininamide by trypsin was reinvestigated. In Fig. 1 are shown the results obtained over a range of substrate concentration from 0.005 to 0.075 M plotted according to zero order reaction kinetics. In this range of substrate concentration the initial reaction course is the same for all substrate concentrations used. This observation suggested that K_m for this system is so much less than the lowest substrate concentration studied that the reaction follows zero order kinetics throughout this range of substrate concentrations and that deviations from zero order kinetics were caused by inhibition of the enzyme by one of the reaction products. The data shown in Fig. 1 could not be resolved by the Lineweaver and Burk equation for competitive inhibition (10), since the value of the ratio K_m/K_I increased as the reaction progressed.[1]

TABLE I

Chemical Analyses of Benzoylarginine and Some Esters of Benzoylarginine

Compound	N calculated	N found	M.p.
	per cent	*per cent*	°C.
BA	20.2	20.1	
BAME·HCl*	16.8	16.4	
BAEE·HCl	16.1	16.0	129 -130
BAIPE·HCl	15.7	15.2	173.5-174.5
BACHE·HCl†	14.1	13.6	182 -183
BABE·HCl	13.9	13.7	75 - 78

* Extremely hygroscopic glass.

† After washing with ether and prolonged drying *in vacuo* the odor of cyclohexanol still clung to this preparation.

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[1] K_m is the Michaelis constant and is defined by $K_m = (k_2 + k_3)/k_1$ where k_1 is the rate constant for the reaction of enzyme and substrate to form the enzyme-substrate complex, k_2 is the rate constant for the reverse reaction, and k_3 is the rate constant for the slowest reaction between the enzyme-substrate complex and the ultimate formation of free enzyme and reaction products. K_I is the enzyme-inhibitor dissociation constant.

Since the addition of an equimolar quantity of ammonium chloride to the substrate resulted in no diminution of the reaction rate, it was concluded that inhibition must be due to benzoylarginine.[3]

Although the true solubility of benzoylarginine is very low, it was observed during the recrystallization of this substance that stable supersaturated solutions could be easily prepared by dissolving benzoylarginine at high temperatures and allowing the solution to cool. The small tendency of benzoylarginine to crystallize from supersaturated solutions probably accounts for the fact that enzymatic reaction solutions remain homogeneous even though the amount of benzoylarginine formed by the hydrolysis of a soluble derivative is much greater than the true solubility of this substance.

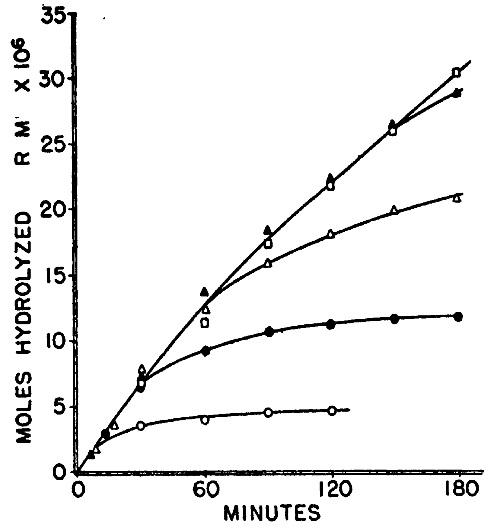

Fig. 1

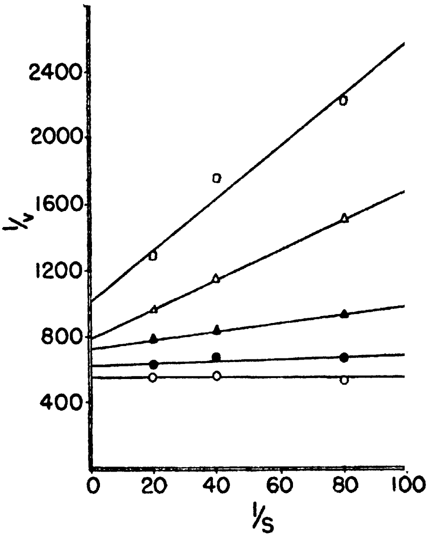

Fig. 2

Fig. 1. Hydrolysis of benzoylargininamide by trypsin. Trypsin concentration, 0.060 mg. of nitrogen per ml. of reaction solution. Determinations made at 25° in 0.1 M phosphate buffer, pH 7.72. BAA concentration is shown by ○ 0.005 M, ● 0.0125 M, △ 0.025 M, ▲ 0.050 M, □ 0.075 M.

Fig. 2. Plot of the Lineweaver and Burk (10) equation for inhibition of tryptic hydrolysis of BAA by benzoylarginine. $1/v$ is the reciprocal of the initial reaction velocity in moles hydrolyzed per liter per minute and $1/S$ is the reciprocal of the initial substrate concentration. Trypsin concentration 0.055 mg. of N per ml. of the reaction solution. Concentration of benzoylarginine is given by ○ none, ● 0.005 M, ▲ 0.0125 M, △ 0.025 M, □ 0.050 M.

This observation was utilized in making the determinations shown in Fig. 2. It is apparent from this plot that in low concentrations of benzoylarginine the inhibition of the hydrolysis of BAA by trypsin is almost entirely non-competitive and that the competitive nature of the inhibition is increased by increasing the concentration of benzoylarginine. Inhibition of this indeterminate type has been previously reported by Elkins-Kaufman and Neurath (12) for the action of butyric acid and chloroacetic acid on the carboxypeptidase-carbobenzoxyglycyl-L-phenylalanine system.

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[3] This result has been independently reached by Harmon and Niemann (11). We are indebted to these authors for access to these data prior to their publication.

Action of Trypsin on Esters of Benzoylarginine—Although it has been observed (1) that the hydrolysis of BAME by trypsin follows zero order kinetics, no attempt has been made to determine whether the range of concentrations in which deviations from zero order kinetics occur is experimentally attainable. With the present method of determining esterase activity, concentrations of substrate below about 5 × 10^{-4} M cannot be used conveniently. Fig. 3 shows the course of the reaction over the concentration range from 0.0007 to 0.03 M. Within this range the reaction apparently follows zero order kinetics through a very large part of the reaction course. From the concentration of substrate remaining when deviations from zero order kinetics occur, it can be estimated that K_m must be smaller than 8 × 10^{-5} M. This very low value of K_m, together with the relative inefficiency of benzoylarginine as an inhibitor in dilute solutions, must account for the difference in apparent order between the enzymatic hydrolysis of BAA and that of BAME.

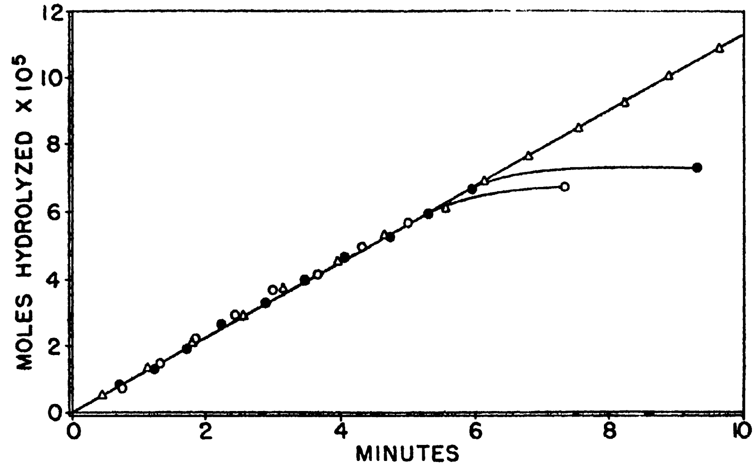

Fig. 3. Hydrolysis of BAME by trypsin at 30°. 0.0334 mg. of trypsin N represents the amount of trypsin used for each determination. 2 ml. of 0.1 M phosphate buffer, pH 7.8, were added to each reaction solution. pH 8.00 was the null point for these measurements. The concentration of BAME and the volume of the reaction solution are shown by ○, 0.0007 M, 100 ml.; ●, 0.003 M, 25 ml.; △, 0.03 M, 5 ml.

In order to evaluate the effect of the size and nature of the alcohol group on the tryptic hydrolysis of esters of benzoylarginine, rate determinations with BAME, BAEE, BAIPE, BACHE, and BABE were made at identical enzyme and substrate concentrations. Fig. 4 shows the results of these determinations at two temperatures. It is apparent that replacement of the methyl group of BAME by a variety of alcohol groups results in no significant change in the hydrolysis rate.[3]

A series of determinations at varying substrate concentrations was made with each ester to determine whether the kinetics of the hydrolysis of any of the esters studied could be characterized by a measurable K_m value. In all cases the results were identical with those shown in Fig. 3 for BAME.

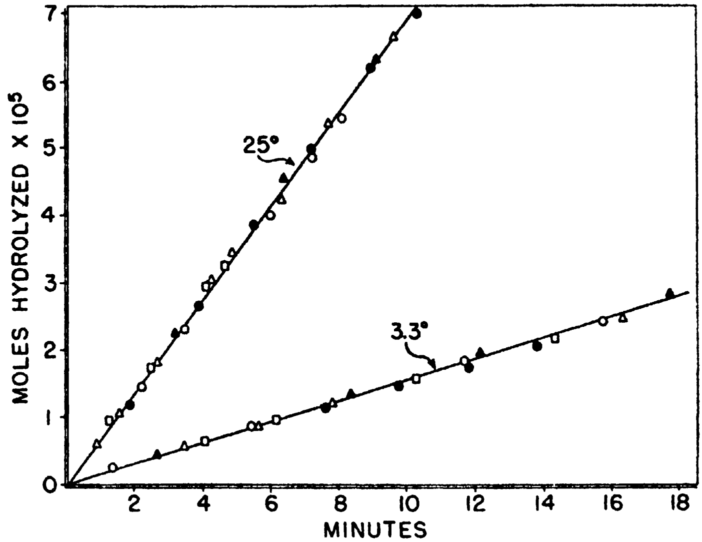

FIG. 4. Hydrolysis of esters of benzoylarginine by trypsin at 25° and at 3.3°. The volume of the reaction solution was 10 ml.; ester concentration 0.0075 M. 0.0227 mg. of trypsin nitrogen present in each reaction solution. The null point for these determinations was pH 8.00. For clarity many points have been omitted. The esters are designated by ○ BAME, ● BAEE, △ BAIPE, ▲ BACHE, □ BABE.

It is generally assumed that enzymatic reactions occur in two steps, a combination between enzyme and substrate and the subsequent activation and hydrolysis of the substrate. When zero order kinetics are found, it is usually postulated that the combination step is so rapid as compared to any subsequent process that the enzyme is always saturated with substrate. Since K_m for the esters of benzoylarginine is so small that the two steps in the reaction cannot be distinguished experimentally, it was reasoned that altering the character of the solvent might so alter the rates of the two processes that they could be measured separately. Although this expectation was not realized, the results obtained are of interest.

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[3] It is of interest that the α-monoglyceride of benzoylarginine, a difficultly characterized oil, prepared by the reaction of the silver salt of benzoylarginine with α-glycerol monochlorohydrin, is also hydrolyzed at a rate identical with that of the other esters.

When the enzymatic hydrolysis of BAME is carried out in ethanolic solutions, the initial reaction rate, up to a limiting concentration of alcohol, is greater than that observed in water.[4] In 16 volume per cent ethanol the initial rate is increased about 35 per cent over that in water, and in 32 volume per cent ethanol the increase is about 50 per cent. At an ethanol concentration of 54 volumes per cent the initial rate is identical with that in water. As the reaction in ethanolic solutions progresses, however, the rate decreases, the decrease being greater the higher the ethanol concentration. Analogous, but less marked, changes in rate were observed when BAA was acted upon by trypsin in ethanolic solutions. These observations are in agreement with the findings of Risley, Buffington, and Arnow (13) for the action of trypsin on bovine serum.

The failure of the esterase reaction to follow zero order kinetics in alcoholic solutions suggested that the enzyme might be inhibited by one of the reaction products in such solutions. Determinations in water and in 16 volume per cent ethanol indicated that the addition of 0.01 M benzoylarginine or of 0.01 M ammonium chloride causes no change in the rate of hydrolysis of 0.01 M BAME. It was also observed that 0.01 M benzoylarginine causes no inhibition in 50 per cent ethanol. Neither 0.01 M arginine hydrochloride nor 0.01 M guanidine hydrochloride causes any rate change in 16 per cent ethanol.

When the rate of hydrolysis of BAME and of BAEE by trypsin was studied in a series of alcohols at the 16 volume per cent level, it was found that methanol causes a 15 per cent increase in rate, ethanol causes a 35 per cent increase, and *n*-propanol and *tert*-butanol cause a rate increase of about 40 per cent over that observed in water. No change in reaction rate was observed when the reaction was studied in 0.1 M glycine.

It has been suggested[5] that the esterase activity of the proteases might be distinguished from the activity of the true esterases by the effect of fluoride upon this activity. Dry trypsin powder was dissolved in 0.5 M sodium fluoride solution and, after 15 minutes, a portion of this solution was added to BAME in the usual buffer. The concentration of fluoride in the reaction solution was 0.025 M. A rate identical with that observed in the absence of fluoride was found.

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[4] The possibility that the acceleration observed in the initial stages of the reaction in 16 and 32 volume per cent ethanol might be due to traces of some activating material was ruled out by using carefully redistilled absolute alcohol for comparative rate studies. The observed rate was independent of the source of the alcohol used.

[5] By Dr. Frederick Bernheim.

DISCUSSION

The data presented in Figs. 1 and 2 show clearly that the hydrolysis of benzoyl-L-argininamide by trypsin cannot be interpreted by a single order of reaction, since one of the reaction products, benzoylarginine, exerts an inhibitory effect on the reaction. This inhibition is of an indeterminate type. It is initially non-competitive, but additional increments of benzoylarginine formed during the reaction cause disproportionately greater inhibition of the enzyme.

These results are in essential agreement with those of Harmon and Niemann (11) which have recently come to our attention. According to their data a value of $K_m = 0.0021$ fits the kinetics of the tryptic hydrolysis of BAA at 25° reasonably well provided values of K_m/K_I varying between 1 and 2 are assumed, the higher value being required to approximate the reaction course, on the basis of competitive inhibition, at higher initial substrate concentrations.

Although the esters of benzoylarginine used for this study probably show some variation in their resistance to non-enzymatic hydrolysis, they are hydrolyzed by trypsin at identical rates. As enzymatic reactions are usually formulated, the possible limiting rates for a zero order reaction are (1) the rate of activation of the enzyme-substrate complex, (2) the rate of entry of water into the hydrolytic process, and (3) the rate of desorption of the products from the enzyme surface. The third of these possibilities is rendered improbable by the observation that only benzoylarginine, a product formed in the hydrolysis of both BAA and of the esters studied here, has been found to have inhibitory activity. Were the rate of desorption of the products the limiting step, the rates of hydrolysis of esters and amides should be identical. The other two possibilities can be distinguished only formally and may occur as one process.

If it is true that the rate being measured in the hydrolysis of esters by trypsin is the rate of activation of the enzyme-substrate complex, then it must be this rate which is increased by the addition of alcohols to the system. It is of interest that for the chymotrypsin-acetyl tyrosinamide system the addition of methanol causes no change in the rate of activation of the enzyme-substrate complex.[6]

The results obtained here at two temperatures for the five esters of benzoylarginine agree well with those previously reported (1) for BAME at three temperatures. Thus, over the range 0.5–42°, ΔE, the Arrhenius activation energy for the rate-limiting step, is 11,200 calories per mole. The absolute reaction rate (moles of ester hydrolyzed per mole of enzyme[7] per second) is 26.7 reciprocal seconds at 25°. With use of the theory of absolute reaction rates (14), $\Delta H^* = 10{,}600$ calories per mole, $\Delta F^* = 15{,}500$ calories per mole, and $\Delta S^* = -16.5$ entropy units. These values are of the same order as those previously reported for the hydrolysis of specific esters by chymotrypsin (2).

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[6] Kaufman, S., and Neurath, H., *J. Biol. Chem.*, in press.

[7] The molecular weight of trypsin is assumed to be 36,000.

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SUMMARY

It has been found that benzoyl-L-arginine inhibits the tryptic hydrolysis of benzoyl-L-argininamide. This inhibition is of indeterminate type, being almost non-competitive in low concentrations of benzoyl-L-arginine and approaching competitive inhibition with increasing concentrations of this reaction product.

The methyl, ethyl, isopropyl, benzyl, and cyclohexyl esters of benzoylarginine are hydrolyzed at identical rates by trypsin. These reactions follow zero order kinetics and none is inhibited by benzoylarginine.

The effect of added alcohols, fluoride, arginine hydrochloride, and guanidine hydrochloride upon the rate of hydrolysis of benzoyl-L-arginine methyl ester by trypsin has been determined.

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THE METABOLISM OF C^{14}-LABELED GLUCOSE BY THE RAT DIAPHRAGM IN VITRO*

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The rat diaphragm was shown by Gemmill (1, 2) to be a satisfactory preparation for the study of carbohydrate metabolism in muscle *in vitro.* It has been employed by Gemmill (3), Hechter, Levine, and Soskin (4), Stadie and Zapp (5, 6), and Verzar and Wenner (7) to study the effect of insulin on glycogen formation, and by Krahl and Cori (8) to study the effect of insulin on glucose disappearance. The present experiments, with C^{14}-labeled glucose as the substrate, were undertaken to determine the relative amounts of glucose converted to glycogen and glucose metabolized to CO_2 by muscle cells and the effect of insulin on these processes. In previous studies (9), the effect of insulin on the metabolism of glucose to carbon dioxide was determined from calculations of the respiratory quotient. In the present experiments, direct determinations of the amount of labeled glucose metabolized to CO_2 have been made and the extent of its conversion to glycogen determined.

The data to be reported include observations on the diaphragms of normal, diabetic, adrenalectomized, hypophysectomized, diabetic-adrenalectomized, and adrenalectomized-hypophysectomized rats.

Materials and Methods

Animals—Most of the animals used in these experiments were male Wistar strain rats, weighing 150 to 200 gm., obtained from the Albino Farms. A few Wistar rats from the Charles River Breeding Laboratories and from our own Wistar colony were used; no differences in these three strains were observed. All animals except those which were adrenalectomized and hypophysectomized were fed on a stock diet of Purina dog pellets.

Diabetes was induced by single intravenous injections of 50 mg. of alloxan per kilo of body weight. The alloxan was administered as a 5 per cent solution in water, freshly prepared. After injection, animals were fed the stock diet and given water to drink. Urine glucose determinations were made every other day until the animals were used. The diabetic animals were kept about 7 days before being used in an experiment.

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Bilateral adrenalectomy was performed in a single operation by means of the Ingle and Griffith (10) technique and ether anesthesia. Both adrenalectomized and diabetic-adrenalectomized rats were fed a synthetic, low carbohydrate diet and were given 1 per cent sodium chloride to drink. Since diabetic-adrenalectomized animals tend to die of hypoglycemia on prolonged fasting, the period of fasting of these animals was limited to 6 to 10 hours.

The hypophysectomies were performed by Dr. Roy O. Greep, of the School of Dental medicine, using the ventral approach. These animals were kept 2 to 10 weeks before being used in the experiments. Some rats were adrenalectomized, given 5 days to recover, then hypophysectomized and kept 4 to 8 days more before being used in experiments. All hypophysectomized rats were fed a special synthetic high fat diet.

Substrate—Glucose labeled with C^{14} in positions 3 and 4 was made biosynthetically *in vitro* as glycogen in liver slices incubated with pyruvate and $C^{14}O_2$ in a potassium-enriched medium.[1] The glycogen was then hydrolyzed for 2 hours with 0.33 N H_2SO_4 and the sulfuric acid removed by the addition of saturated $Ba(OH)_2$.

Glucose uniformly labeled in all six carbons was made photosynthetically as starch in bean leaves in an atmosphere of $C^{14}O_2$. 5 to 6 week-old bean seedlings were kept in the dark 4 days to exhaust the starch reserves in the leaves. A vacuum desiccator was prepared by placing 10.6 gm. of $BaC^{14}O_3$ recovered as wastes from respiratory experiments in the bottom in a little water to facilitate mixing. (The activity of the $BaC^{14}O_3$ was about 45,000 counts per minute per mM of carbon.) About 20 gm. fresh weight of bean leaves were removed from the stalks and placed in CO_2-free water in small beakers on the desiccator plate and held in place by scotch tape. 20 ml. of 9 N perchloric acid was placed in a round-bottom 50 ml. centrifuge tube in one of the holes in the desiccator plate. The top was put on, the desiccator evacuated to 50 mm. of Hg pressure, and the centrifuge tube was tipped by a blow on the side of the desiccator, thereby mixing the acid with the $BaC^{14}O_3$ and releasing $C^{14}O_2$. The desiccator was kept at reduced pressure for 1 hour, after which CO_2-free air was admitted to bring the pressure to slightly below atmospheric pressure. The desiccator was then put in the light for 48 hours. At the end of this photosynthetic period, the desiccator was evacuated five times through an NaOH train of absorbers to recover the CO_2 remaining. After the last evacuation, 50 ml. of 5 N NaOH were drawn into the desiccator to neutralize the perchloric acid and to absorb any remaining CO_2.

The leaves were removed, ground in a Waring blendor in 200 ml. of water for 5 minutes, and strained through cheese-cloth. The residue was ground for 5 minutes in an additional 75 ml. of water and strained, and this second residue was ground a third time in 75 ml. of water. The combined filtrates were centrifuged 10 minutes at 10,000 R.P.M. The several sediments from this centrifuging were combined into a single 50 ml. centrifuge tube and again centrifuged. The sediment consisted of three parts: a bottom layer of pale green cell fragments, a middle layer of white starch granules, and a thin upper layer of very dark green chloroplasts. These layers were separated by differential centrifuging (11), the chlorophyll extracted by 3:1 alcohol-ether, and the residual starch placed in boiling 30 per cent KOH for 20 minutes. It was then precipitated from solution by the addition of 3 volumes of alcohol and centrifuged. The resulting starch was hydrolyzed with 0.33 N H_2SO_4 for 2 hours to yield glucose. The sulfuric acid was removed by the addition of saturated $Ba(OH)_2$ in the presence of phenol red. $Ba(OH)_2$ was added to slight alkalinity. The $BaSO_4$ was removed by centrifugation and dilute phosphoric acid (0.01 M) added dropwise to neutrality, thereby removing the excess of Ba^{++}. The resulting solution of glucose was used without further purification.

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[1] Topper, Y. J., personal communication.

Suspension Medium—The suspension medium in which the diaphragm muscle was incubated was identical with that used by Stadie and Zapp (6): 0.040 M sodium phosphate, 0.005 M $MgCl_2$, 0.08 M NaCl, and 200 mg. per cent of glucose, initial pH (by glass electrode) 6.8. The insulin used was commercial (20 units per ml.) iletin (insulin, Lilly). The final concentration of the insulin in the incubation medium was 0.5 unit per ml., 1.5 units per incubation vessel.

Procedure—The animals were killed by a blow on the head, the abdomen was opened, and the diaphragm was exposed. Each hemidiaphragm was removed with minimum trauma and bleeding, trimmed to remove the central tendon, and blotted on filter paper. A small piece of each hemidiaphragm was weighed, placed in boiling 30 per cent KOH, and analyzed for initial glycogen. The major part of the hemidiaphragm, about 100 mg. in weight, was weighed on a torsion balance and placed in a Warburg vessel in 3 ml. of incubation medium. One hemidiaphragm from each rat was placed in a vessel with insulin and one in a vessel without insulin. In the center well were placed a piece of hard filter paper and 0.2 ml. of 5 per cent NaOH, prepared CO_2-free by dilution from saturated NaOH.

The vessels were attached to Warburg manometers, gassed with 100 per cent oxygen for 10 minutes, and then incubated for 2 hours at 38°, while being shaken at 120 cycles per minute. At the end of the 2 hour period, the vessels were removed, the alkali-soaked filter paper from the center well (containing CO_2 and $C^{14}O_2$) was placed in 8 ml. of CO_2-free water in a centrifuge tube, the center well was rinsed three times with CO_2-free water, and the washings added to the contents of the centrifuge tube. The tubes were then stoppered and allowed to stand for half an hour, after which the filter paper was pressed dry against the side of the tube and removed. Control experiments showed that all of the CO_2 in the center well was recovered by this procedure. 1 ml. of 0.11 M $BaCl_2$ was added, and the $BaCO_3$ precipitated and centrifuged, washed with CO_2-free water and 95 per cent ethanol, plated on stainless steel cups, and counted by an end window Geiger tube.

The possibility remained that some significant fraction of the glucose was metabolized to CO_2 in the 10 minute period at the beginning of the experiment in which the vessels were gassed with oxygen. To test this, four experiments were performed in which the vessels were filled with the incubation medium and center well alkali and gassed before the muscle was added. After a 10 minute gassing period, the vessels were removed momentarily from the manometers, the diaphragms added, and the vessels replaced on the manometers immediately so that all the CO_2 evolved while the diaphragms were in the incubation medium was trapped. Essentially no difference was found between these experiments and those done in the usual way. The average amount of CO_2 derived from the labeled glucose of the medium was 13 per cent without added insulin and 16 per cent with added insulin in these experiments, compared to 10 per cent without added insulin and 16 per cent with added insulin in the experiments performed the usual way.

Tests were made for the completeness of the transfer of CO_2 from the incubation medium to the center well in three ways. In eight experiments, 0.2 ml. of 1 N H_2SO_4 was placed in the side arm and dumped into the medium at the end of the experiment to bring the final pH of the medium to 1.8. There was no significant difference in the total amount of CO_2 recovered nor in the total counts of the recovered CO_2 compared with other experiments in which no acid was added at the end. In two other experiments, the medium at the end was analyzed for its CO_2 content by the Van Slyke manometric method. This was found to be 0.00018 and 0.0003 mM of CO_2 per incubation vessel, respectively, and amounted to 1.0 and 1.9 per cent of the total CO_2 recovered. Another check is provided by calculating the CO_2 evolved during the experiment from the weight of $BaCO_3$ recovered and then calculating the R.Q. from this value and the oxygen uptake which was measured directly during the incubation period. If any significant amount of CO_2 remained in the medium, the R.Q. so calculated would be less than 1; yet in these experiments the R.Q. varied from 1.0 to about 1.1. The amount of CO_2 present in the solution plus tissue at zero time was less than could be determined by weighing the $BaCO_3$ from the center well. The CO_2 initially present in the tissue (10 mM per kilo of muscle) would yield 0.02 to 0.03 mg. of $BaCO_3$. Control experiments, in which muscle was incubated anaerobically, resulted in no detectable amount of CO_2 (measured as $BaCO_3$) in the center well.

The diaphragm was removed from the vessel, placed in 1 ml. of boiling 30 per cent KOH in a 15 ml. centrifuge tube, and analyzed for glycogen by the method of Good, Kramer, and Somogyi (12). The glycogen was precipitated from the alkali by 1.5 volumes of alcohol. The tube was placed in an ice bath to insure complete precipitation, then centrifuged, and the supernatant discarded. The glycogen was resuspended in 1 ml. of water, precipitated by 1.5 ml. of alcohol, boiled, and placed in an ice bath, then centrifuged. The supernatant was discarded, the glycogen taken up in 0.5 ml. of water, 0.5 ml. of 10 N H_2SO_4 was added, and the tube was placed in a boiling water bath for 30 minutes. It was removed, cooled, neutralized with 5 N NaOH (phenolphthalein), 1 drop of 2 N H_2SO_4 was added, and then it was made up to 3 ml. with water. A 1 ml. aliquot was analyzed for glucose by the Nelson method (13). In eight experiments, aliquots of this glucose obtained from the hydrolysis of the glycogen were oxidized by the wet combustion technique of Van Slyke and Folch (14), and the resulting CO_2 was precipitated as $BaCO_3$, plated, and analyzed for C^{14}. The incubation medium was pipetted from the Warburg flasks and aliquots were analyzed for residual glucose by the Nelson method (13), with $Ba(OH)_2$ and $ZnSO_4$ to remove interfering substances. The final pH of the medium was measured by the glass electrode and was found to be pH 6.8 ± 0.1.

Blood glucose determinations were made at the time the animals were sacrificed and averaged 98.7 mg. per cent for normal animals, 415 mg. per cent for diabetic animals, 68.3 mg. per cent for adrenalectomized animals, 164 mg. per cent for diabetic-adrenalectomized, 97 mg. per cent for hypophysectomized, and 70 mg. per cent for adrenalectomized-hypophysectomized animals.

Results

Effect of Insulin on Disappearance of Glucose from Medium—In all groups of animals, normal, diabetic, adrenalectomized, diabetic-adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized, the addition of insulin increased the amount of glucose disappearing from the incubation medium (Table I). This confirms the work of Krahl and Cori (8) on normal, diabetic, and adrenalectomized rats. When no insulin is added, the glucose disappearance in the presence of muscle from diabetic rats is lower and that from adrenalectomized rats is greater than in the presence of muscle from normal rats. The glucose disappearance in

TABLE I

In Vitro Utilization of C^{14}-Labeled Glucose by Rat Diaphragm Muscle

	Normal		Diabetic		Adrenalectomized		Diabetic-adrenalectomized		Hypo-physectomized		Adrenalecto-mized-hypo-physectomized	
	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin
1. Total glucose disappearing from medium, *mg. per gm. per hr.*												
Gemmill and Hamman	1.89	3.44										
Stadie	1.91	3.48										
Krahl and Cori	1.93	2.47	0.80	1.38	2.03	2.40	2.18	2.88				
Present experiments*	1.53	2.87	0.78	2.17	2.29	3.91	3.07	5.20	2.55	4.42	3.25	6.11
	±0.046	±0.047	±0.035	±0.10	±0.063	±0.077	±0.084	±0.123	±0.052	±0.079	±0.052	±0.069
No. of animals	14	14	7	7	12	12	10	10	7	7	3	3
2. Glucose metabolized to CO_2,* *mg. per gm. per hr.*	0.11	0.22	0.08	0.11	0.17	0.29	0.15	0.18	0.19	0.27	0.32	0.44
	±0.012	±0.026	±0.019	±0.030	±0.030	±0.056	±0.024	±0.023	±0.038	±0.031	±0.016	±0.021
3. Glycogen synthesized,* *mg. per gm. per hr.*	0.17	0.62	0.14	0.61	0.08	0.54	0.26	0.71	0.76	1.71	1.19	2.23
	±0.062	±0.13	±0.045	±0.19	±0.034	±0.15	±0.084	±0.13	±0.22	±0.43	±0.50	±0.55
4. Glucose unaccounted for, *mg. per gm. per hr.*	1.25	2.03	0.56	1.45	2.04	3.03	2.66	4.31	1.60	2.44	1.74	3.44
5. Utilized glucose recovered as CO_2, %	7.2	7.7	10.3	5.1	7.4	7.4	4.9	3.5	7.5	6.1	9.8	7.2
6. Utilized glucose converted to glycogen, %	11.1	21.6	18.0	28.1	3.5	13.8	8.5	13.6	29.8	38.7	36.6	36.5
7. CO_2 in center well derived from glucose of medium, %	10.4	16.3	8.1	9.2	15.0	25.3	13.8	15.9	22.3	28.5	23.0	33.9

* The values given are means ± standard error.

the presence of diaphragm from diabetic-adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized rats is greater than normal. The figures obtained by Gemmill and Hamman (3), Stadie (5), and Krahl and Cori (8), each of whom used slightly different incubation media, are given for comparison. In our experiments, the decrease in glucose utilization is roughly proportional to the severity of the diabetes. The data given in Table I are for those animals with a fasting blood glucose of 300 mg. per cent or more. Five animals with a lower fasting blood glucose showed no decrease in glucose utilization: 1.53 mg. of glucose per gm. of diaphragm per hour without insulin and 5.14 mg. per gm. per hour with added insulin.

Amount of Glucose Carbon Recovered in CO_2—The amount of glucose metabolized to CO_2 was calculated from the formula: glucose metabolized to CO_2 = ((total counts of C^{14} in CO_2)/(total counts of C^{14} in glucose)) × total amount of glucose in the vessel.

When no insulin was added to the medium, the amount of glucose metabolized to CO_2 by the diaphragm paralleled the total amount of glucose disappearing from the medium. This was true for all six groups of animals. It was less than normal in diabetic and greater than normal in the other experimental conditions (Line 2, Table I). The addition of insulin increased the amount of glucose metabolized to CO_2 by muscle from normal, adrenalectomized, diabetic-adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized rats. These increases were shown to be significant by the "*t* test," which gave P values of less than 0.05 or 0.01 (Table II). The addition of insulin to diabetic muscle caused only a slight increase, one which is not statistically significant (P = about 0.35).

Amount of Glycogen Synthesized—The amount of glycogen synthesized in the muscle during the incubation period (Line 3, Table I) was calculated by subtracting the initial glycogen content from the glycogen present at the end of the experiment. Muscle from both the diabetic and the adrenalectomized animals formed less glycogen than normal muscle, but that from diabetic-adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized animals formed more glycogen (Line 3, Table I). In normal muscle, the addition of insulin caused an 87 per cent increase in glucose utilization, a 100 per cent increase in glucose carbon recovered as CO_2, but a 265 per cent increase in glycogen synthesis.

Glucose Unaccounted for—The glucose carbon recovered as CO_2 and the glycogen synthesized (Lines 5 and 6, Table I) accounted for from 11 to 46 per cent of the total glucose disappearing from the medium; the rest was unaccounted for in these experiments. Some of the glucose unaccounted for is converted to lactate, some to fat, some to protein, and some

TABLE II

Tests For Significance of Insulin Stimulation

	Normal			Diabetic			Adrenalectomized			Diabetic-adrenalectomized			Hypophysectomized			Adrenalectomized-hypophysectomized		
	t	*df*	*P*	*t*	*df*	*P*	*t*	*df*	*P*	*t*	*df*	*P*	*t*	*df*	*P*	*t*	*df*	*P*
Pair comparisons, difference with and without insulin																		
Glucose disappearing from medium..	6.72	13	<0.01	2.45	6	<0.05	3.86	11	<0.01	4.50	9	<0.01	4.56	6	<0.01	7.73	2	<0.05
" metabolized to CO_2..........	5.78	13	<0.01	1.57	6	0.35	3.34	11	<0.01	4.63	9	<0.01	3.98	6	<0.01	5.45	2	<0.05
Glycogen synthesized...............	5.34	13	<0.01	3.35	6	<0.05	3.80	11	<0.01	5.63	9	<0.01	3.39	6	<0.05	6.22	2	<0.05
Tests for significance of difference from normal																		
Glucose disappearance..............				3.26	19	<0.01	3.04	24	<0.01	6.69	22	<0.01	2.37	19	<0.05	3.74	15	<0.01
" metabolized to CO_2..........				1.31	19	>0.05	1.88	24	>0.05	1.54	22	>0.05	2.29	19	<0.05	6.36	15	<0.01
Glycogen synthesized...............				0.33	19	>0.50	1.18	24	>0.05	0.85	22	<0.50	10.35	19	<0.01	11.08	15	<0.01

is present at the end of the incubation period as the various intermediates between glucose and the end-products. Experiments are under way to determine the fraction of the glucose converted to each of these substances.

The increase in the amount of glucose metabolized to CO_2 when insulin was added paralleled the increase in the total glucose utilized; so that the same fraction of the glucose utilized, about 7 per cent, was oxidized with or without insulin (Line 5, Table I). However, the increase in the amount of glycogen synthesized when insulin was added was greater than the increase in the total glucose uptake, so that there was an increase in the fraction of the glucose uptake which was converted to glycogen (Line 6, Table I). If we assume that insulin acts on the hexokinase reaction and increases the amount of glucose-6-phosphate present, these facts suggest that the limit of the rate at which glucose-6-phosphate can be passed through the Embden-Meyerhof cycle to pyruvate was lower in our experiments than the limit of the rate at which glucose-6-phosphate can be converted into glucose-1-phosphate and glycogen.

Fraction of Center Well CO_2 Derived from Labeled Glucose—The fraction of CO_2 collected in the center well derived from the glucose of the medium was calculated by dividing the specific activity (counts per minute per mM of carbon) of the CO_2 by the specific activity (counts per minute per mM of carbon) of the glucose. Since the activity of the glucose was measured by combusting it to CO_2 and plating the CO_2 as $BaCO_3$, the specific activity is the average activity of all 6 carbons in the glucose molecule, although actually only carbons 3 and 4 are radioactive. These calculations show that in muscle from normal, adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized rats the addition of insulin increased not only the amount of glucose metabolized to CO_2, but also the fraction of CO_2 in the center well derived from the labeled glucose of the medium. There was, however, no over-all increase on the addition of insulin in the rate of respiration as measured by the oxygen uptake. This would indicate that when insulin was added the muscle metabolized more glucose and less of other substances. In muscle from diabetic and diabetic-adrenalectomized rats, the addition of insulin had less effect on the fraction of CO_2 derived from the glucose of the medium. In these calculations the assumption is implicit that insulin does not increase the size of the carbohydrate pool (and hence dilute the C^{14} from the glucose) by any route other than that from the glucose of the medium. If insulin does increase the size of the pool of precursors of the Krebs cycle other than those derived from the medium glucose, these calculations based on specific activity will be invalid. However, neither the Q_{O_2} nor the R.Q. is changed by the addition of insulin.

Glycogen Synthesis—The percentage of the glycogen synthesized in the diaphragm during the incubation period derived from the glucose of the medium was calculated by comparing the specific activity of the glycogen synthesized with the specific activity of the glucose in the medium. In seven out of eight experiments, the glycogen synthesized in the presence of a medium containing 200 mg. per cent of glucose apparently did not come entirely from the glucose of the medium. In three experiments with muscle from normal rats incubated in the absence of insulin, the fraction of new glycogen formed during the incubation period derived from tagged glucose was 0.26, 0.79, and 1.0. In five experiments with muscle from normal rats incubated in the presence of insulin, the fraction of new glycogen formed during the incubation period derived from tagged glucose was 0.53, 0.56, 0.60, 0.76, and 0.24. When muscle is incubated in the absence of insulin, the amount of glycogen synthesized is small compared to that present initially; hence the correction factor, for converting counts per minute per mg. of total glycogen present at the end to counts per minute per mg. of glycogen synthesized, is large and great differences in the value of the fraction derived from glucose occur. But when muscle is incubated in the presence of insulin, the amount of glycogen synthesized is large, the correction factor is smaller, and the values of the fraction of glycogen synthesized derived from the glucose of the medium are less variable.

Evidence that glycogen of diaphragm muscle can be derived from precursors other than glucose was obtained by the use of α-carbon-labeled pyruvate ($CH_3C^{14}OCOO^-$) and carboxyl-labeled acetate ($CH_3C^{14}OO^-$) as substrates. In six experiments with C^{14}-pyruvate as substrate (glucose absent), the average amount of new glycogen derived from the pyruvate was estimated as 13.1 per cent when insulin was absent, and 11.6 per cent when insulin was present. In four experiments with C^{14}-acetate (glucose absent), the amount of glycogen formed in the muscle in a 2 hour incubation period from the acetate of the medium was always less than 0.2 per cent (Table III).

Metabolism of 3,4-Labeled and Uniformly Labeled Glucose—In most of the experiments presented here, 3,4-labeled glucose was used as the substrate. That this material was eventually metabolized completely to CO_2 and water, and not to some 2 carbon fragment which releases the C^{14} from positions 3 and 4, is shown by the fact that exactly similar results were obtained when uniformly labeled glucose hydrolyzed from bean starch was used. In four paired experiments, in which uniformly labeled glucose was used as the substrate, the average values of the glucose metabolized to CO_2 by normal muscle were 0.15 mg. per gm. per hour without insulin and 0.29 with added insulin. In the ten paired experiments with 3,4-labeled glucose, the average values of the glucose metabolized to CO_2 by normal muscle were 0.10 and 0.19 mg. per gm. per hour respectively, whereas if the 3 and 4 carbons had been metabolized at a significantly greater rate than the other carbons of glucose, the CO_2 values obtained with 3,4-labeled glucose should have been significantly higher than those obtained with uniformly labeled glucose.

High Concentrations of Glucose—A few experiments were performed in the presence of glucose concentrations of 300 and 500 mg. per cent. The rate of glucose utilization in both normal and diabetic muscle was increased when the concentration of the glucose in the medium was increased, but even at the higher glucose concentrations, insulin had the effect of increasing the glucose utilization (Table IV). Although the amount of glucose metabolized to CO_2 by normal muscle was not significantly modified by increasing the concentration of glucose in the medium, insulin still produced

TABLE III

In Vitro Synthesis of Glycogen from Pyruvate and Acetate by Rat Diaphragm Muscle

Substrate	Per cent glycogen synthesized, derived from labeled substrate	
	Without insulin	With insulin
$CH_3C^{14}OCOO^-$	18.2	16.6
	9.9	13.5
	11.2	4.6
Average	13.1	11.6
$CH_3C^{14}OO^-$	0.21	0.13
	0.10	0.08
	0.15	0.00
Average	0.15	0.07

TABLE IV

Effect of Variations in Concentration of Glucose in Incubation Medium on Utilization in Vitro of Glucose by Rat Diaphragm Muscle

The results are expressed as mg. per gm. per hour.

	Normal rats		Diabetic rats		Normal rats		Diabetic rats		Diabetic rats	
	200 mg. glucose per 100 ml. medium				300 mg. glucose per 100 ml. medium				500 mg. glucose per 100 ml. medium	
	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin	Without insulin	With insulin
No. of experiments	14	14	7	7	2	2	2	2	2	2
Total glucose uptake	1.60	2.82	1.03	2.37	5.57	9.72	8.45	9.77	9.87	18.10
Glucose metabolized to CO_2	0.14	0.24	0.09	0.11	0.12	0.26	0.12	0.21	0.17	0.21
Glycogen synthesized	0.21	0.70	0.15	0.63	0.45	0.76	0.13	0.68	0.10	0.76
Glucose unaccounted for	1.25	1.88	0.79	1.63	5,00	8.70	7.90	8.88	9.60	17.13

an increase in glucose metabolism. Insulin did not cause a significant increase in the amount of glucose from the medium recovered as CO_2 when diabetic muscle was incubated in any of the glucose concentrations tried. The amount of glycogen synthesized by normal muscle with or without insulin was greater when the concentration of glucose in the medium was increased, but insulin still had an effect on glycogen synthesis at a level of 300 mg. per cent of glucose in the medium. In contrast, the amount of glycogen synthesized by diabetic muscle in the absence of insulin was not increased as the glucose of the medium was increased, whereas the amount synthesized in the presence of insulin was increased slightly as the concentration of glucose in the medium was raised. At all glucose concentrations studied, insulin had a marked effect in increasing glycogen synthesis by diabetic muscle.

DISCUSSION

Although more questions have been raised than answered by the experiments described above, it is of interest to list the inferences which may be drawn from them. For comparison, the results obtained in the several experiments are collected in Fig. 1.

First, it would appear that even in the excised rat diaphragm a metabolic pool of glycogen precursors exists due to the finding that the specific activity of the glycogen carbon and of the carbon of the CO_2 recovered in the center well was less than the specific activity of the glucose carbon.

Second, the effect of insulin *in vitro* is consistent with the Cori demonstration that insulin activates hexokinase. The evidence presented for this rests on the increase in glucose utilization and in muscle glycogen resulting in all experiments upon the addition of insulin. In addition, insulin produced an increase in the amount of tagged glucose recovered as CO_2 in all experiments except those on muscle from diabetic animals. From this, it might be inferred that in all save the diabetic animals metabolism of glucose-6-phosphate to both glycogen and to CO_2 was facilitated due to a speeding up in its rate of formation. The reason for the absence of an increase in metabolic CO_2 from glucose in the diabetic animals is unknown and presents an interesting question worthy of further study.

Third, the absence of the hypophysis resulted in an increase in glucose utilization, glycogen formation, and in the amount of glucose metabolized to CO_2. By themselves, these observations would be consistent with the Cori experiments that hormones of the hypophysis and adrenals inhibit hexokinase and that insulin can neutralize this inhibition. However, this explanation would seem to be inadequate when we consider that insulin increased carbohydrate utilization, glycogen formation, and the amount of glucose metabolized to CO_2 in muscle from animals which had been both

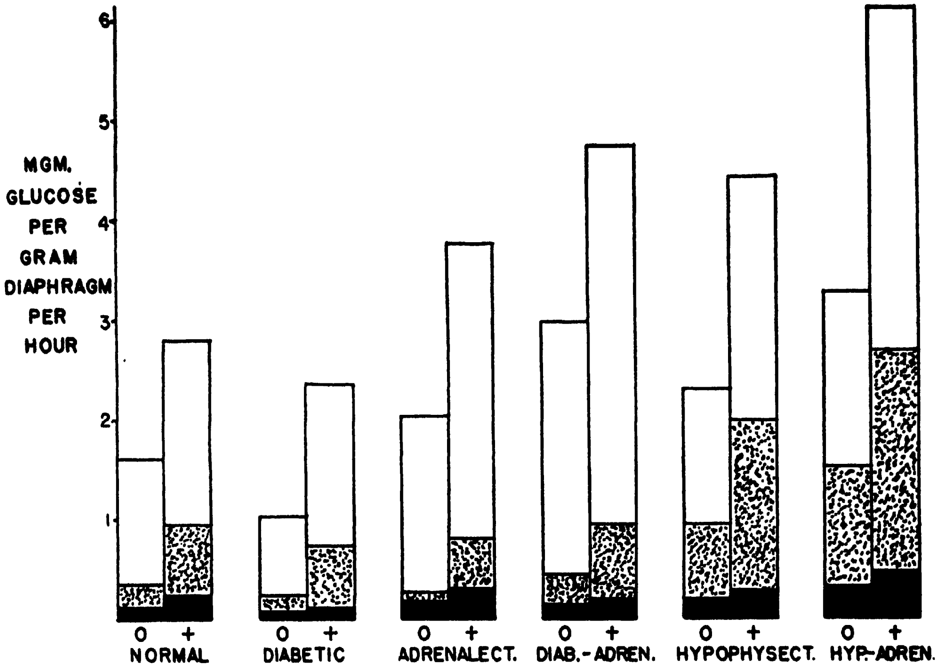
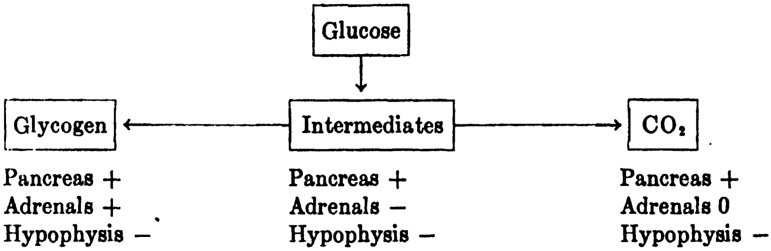


FIG. 1. The effect of insulin on the utilization of glucose by isolated diaphragm muscle. 0 = no insulin added, + = insulin added, 0.5 unit per ml. The height of the bar represents the amount of glucose disappearing from the medium; the black portion represents the fraction metabolized to carbon dioxide, the stippled portion the fraction synthesized to glycogen, and the white portion the fraction unaccounted for in these experiments.



+ designates an increase produced by the presence of the specified hormones, and − designates a decrease produced by their presence. The term "intermediates" designates glucose-6-phosphate and all intervening compounds between glycogen and CO_2.

adrenalectomized and hypophysectomized. It is pertinent that Krahl and Park (15) and Perlmutter and Greep (16) found undiminished insulin effects on glucose utilization in isolated diaphragms from hypophysectomized rats.

Fourth, the absence of the adrenals resulted in an increase of glucose utilization, but a decrease in glycogen formation. This could be accounted for by an inhibitory action of adrenal hormones on some enzymatic step between glucose-6-phosphate and CO_2 or by an acceleration of some side reaction, such as the reduction of pyruvate to lactate.

Though avowedly an oversimplification, the observations presented above may be qualitatively expressed in the accompanying diagram.

It remains for further study to bring more specific knowledge of the sites of action of the hormones of the pancreas, hypophysis, and adrenal glands. For the present, the Cori hypothesis would seem to be adequate if one includes an additional inhibitory action by adrenal hormones at some point between glucose-6-phosphate and CO_2.

We wish to express our thanks to Dr. A. K. Solomon of the Biophysical Laboratory for performing the C^{14} analyses and to Miss Kathleen Whitehouse for her technical assistance.

SUMMARY

1. The glucose disappearance, glycogen synthesis, and metabolism of glucose to carbon dioxide by isolated rat diaphragm have been measured in muscle from normal, diabetic, adrenalectomized, hypophysectomized, adrenalectomized-hypophysectomized, and diabetic-adrenalectomized rats. In all types of animals, the addition of insulin increased the amount of glucose disappearing from the medium and the amount of glycogen synthesized. Insulin increased the amount of glucose carbon appearing in carbon dioxide in all but the diabetic diaphragm, in which the increase was not significant. When no insulin was added to the medium, the glucose utilization and glucose metabolized to carbon dioxide were lower than normal in muscle from diabetic rats and greater than normal in muscle from adrenalectomized, hypophysectomized, and adrenalectomized-hypophysectomized rats. Glycogen synthesis when no insulin was added was lower than normal in muscle from diabetic and adrenalectomized rats and greater than normal in muscle from hypophysectomized, diabetic-adrenalectomized, and hypophysectomized-adrenalectomized rats.

2. The percentage of the glycogen synthesized by the diaphragm derived from the glucose of the medium varied from 24 to 100 per cent. The diaphragm can synthesize glycogen *in vitro* from precursors other than glucose: in experiments with α-labeled pyruvate as the substrate, an average of 13 per cent of the new glycogen came from the pyruvate; in experiments with carboxyl-labeled acetate, however, the glycogen formed from acetate was less than 0.2 per cent.

3. The individual carbon atoms of the glucose molecule are metabolized

to carbon dioxide at essentially the same rate, for the same results were obtained with either 3,4-labeled glucose or uniformly labeled glucose as the substrate.

4. The rate of glucose utilization in both normal and diabetic muscle was increased when the concentration of glucose in the medium was increased, and the addition of insulin increased the glucose utilization at all glucose concentrations used.

5. The relation of these observations to the Cori hypothesis of the action of insulin is discussed.

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THE INCORPORATION OF LABELED LYSINE INTO THE PROTEINS OF GUINEA PIG LIVER HOMOGENATE*

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When C^{14}-labeled lysine is incubated with guinea pig liver homogenate, α-aminoadipic, α-ketoadipic, and glutaric acids are formed from the lysine (1). These transformations were established by finding the radioactivity of the C^{14} tracer in the metabolic products. The homogenate proteins coagulated by boiling at pH 5 also contained radioactivity. The counts given by the proteins corresponded to about 0.02 to 0.03 per cent of that added as lysine; the extent of lysine incorporation into the proteins was of the same order of magnitude as Melchior and Tarver (2) had found after incubating S^{35}-labeled methionine and Winnick *et al.* (3, 4) C^{14}-labeled glycine with rat tissue homogenates. Yet we could not satisfy ourselves that the radioactivity remaining in the proteins in our experiments, although it persisted through exhaustive extraction, did not come from traces of adsorbed radioactive lysine. Some counts were found in the protein when the homogenate was boiled prior to incubation with isotopic lysine.

The practical solution to the problem, it seemed, was to find experimental conditions in which very much more of the radioactivity added as lysine would remain in the protein after thorough washing, and little or no radioactivity in the protein of the controls; then we could conclude that the lysine was incorporated into the protein molecule and not adsorbed. The question of the mode of linkage would remain open.

Eventually two sets of conditions were found in which relatively large amounts of labeled lysine are incorporated into the proteins. In the one case, with the whole homogenate as the enzyme system, the optimum pH is in the neighborhood of 6.1, and calcium is required, the optimum concentration being above 0.003 M; the reaction proceeds hardly at all without the addition of calcium. In the other case the enzyme system was the centrifugate obtained by centrifuging the diluted homogenate at 2500*g*; we

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* This work is a part of that done under contract with and joint sponsorship of the Office of Naval Research, United States Navy Department, and the United States Atomic Energy Commission. The C^{14} used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

shall refer to it as the sedimented fraction. The favorable condition when the sedimented fraction is used is at about pH 7.3; it is nearly inoperative at the optimum pH region (6.1) of the whole homogenate; it is not dependent on added calcium, though it is accelerated a little by calcium added to a final concentration of 0.004 M. The evidence points to either two different reactions or to two enzyme systems or substrates which incorporate labeled lysine.

In 2 hours at 38° in the whole homogenate reaction at pH 6.1 with a calcium concentration above 0.003 M, 3 per cent of the added radioactivity was incorporated into the mixed proteins, and 0.22 per cent of the lysine in the proteins was labeled. When the sedimented fraction was used at pH 7.3, in 2 hours at 38° 3 to 5 times as much labeled lysine were incorporated into the proteins as with the whole homogenate. These figures correspond to rates respectively 60 and more than 200 times faster than any reported hitherto on the incorporation of a labeled amino acid into the proteins of tissue homogenate (2–4). It is at the lower limit of the values found when tissue slices (2, 5–7), tissue segments (8), or resting bacteria (9) were incubated with labeled amino acids. Weissman and Schoenheimer (10) found after feeding labeled lysine to the rat that in 4 days 13.4 per cent of the lysine in the proteins was derived from the isotope. This rate of 0.14 per cent per hour is about the same as in the guinea pig liver whole homogenate under our best conditions.

Preparations

L- and D-lysine labeled with C^{14} in the ϵ position were prepared by methods described in a previous communication (1). These were diluted before use with the normal (*i.e.* not radioactive) isomers.

Procedure

The livers used in these experiments were taken from commercially procured adult guinea pigs, kept without food for 20 hours before use, killed by stunning, and bled thoroughly. The liver was washed in ice-cold saline and, without dilution with saline, minced in a Waring blendor for 1½ minutes, then homogenized in the apparatus of Potter and Elvehjem (11), and finally strained through two layers of cheese-cloth. By using undiluted homogenate a relatively large amount of tissue could be contained in a small volume of reaction mixture (0.5 ml. was quite convenient) and we could thus economize on labeled lysine.

The sedimented fraction was prepared as follows: the homogenate was suspended in 15 times its volume of ice-cold Krebs-Henseleit Ringer's solution (12) modified to contain twice the amount of bicarbonate. It was centrifuged in a refrigerated centrifuge at 500*g* for 3 minutes, and the supernatant solution decanted off and centrifuged at 2500*g* for 15 minutes. The resulting supernatant solution was discarded, and the sediment was resuspended in a volume of the modified Ringer's solution equal to that of the diluted homogenate originally used and again centrifuged at 2500*g* for 15 minutes.

The viscous sediment was loosened and mixed and used as such or after mixing with a saline-bicarbonate solution. This fraction contained nuclei, mitochondria, submicroscopic granules, red cells, and probably some débris (13). We intend later to study the incorporation of lysine (labeled) into the separate nuclear, mitochondrial, submicroscopic granular, and cytoplasmic fractions.

The reaction mixtures were made up in Krebs-Henseleit Ringer's solution modified as indicated in the protocols.

The reaction mixtures were made up in 20 ml. Pyrex beakers and incubated at 38° in the apparatus of Dubnoff (14). At the end of an experimental run the contents of each beaker were transferred to a 250 ml. beaker with 80 ml. of water, the small clumps broken with a stirring rod, and then 20 ml. of 35 per cent trichloroacetic acid added. After standing overnight at room temperature the precipitated protein was washed either by filtration or by repeated centrifugation. Both methods gave the same results. In the filtration procedure the protein was filtered with suction onto a weighed circle of filter paper on a coarse sintered glass filter. After all the solution had passed through, the protein remaining on the filter was washed with six 50 ml. portions of 7 per cent trichloracetic acid, sucked dry after each addition, and then washed with three 50 ml. portions of water; the water was then removed by washing three times with 95 per cent ethanol followed by ether. The proteins were dried in a vacuum desiccator over solid NaOH. The centrifugation procedure was as follows. The next day, after the protein had been precipitated with 7 per cent trichloroacetic acid, most of the clear supernatant solution was removed by gentle suction. The remaining suspension was transferred to a weighed 15 ml. thick walled test-tube and centrifuged. The supernatant solution was poured off, and the sediment resuspended and broken up in about 12 ml. of 7 per cent trichloroacetic acid and centrifuged again. This was repeated nine times with trichloroacetic acid and twice with acetone. The protein remaining in the test-tube was then dried in an air-bath at 55°. When there are many protein samples to be washed, the latter procedure is less time-consuming and requires less trichloroacetic acid. The efficacy of either washing procedure is attested to by the very low or negative radioactivity of the proteins in the controls and when certain inhibitors were used.

The protein, after it was dry, was weighed and ground to a fine powder.

For the measurement of its radioactivity 20 mg. were spread evenly on an aluminum plate over a circle 19 mm. in diameter marked on it. Toward the end of the work reported here the circle was cut into the plate as a shallow depression; the spreading of the protein was easier and more even. The thickness of the counting samples was thus 7.05 mg. per sq. cm. The method of measuring the radioactivity was the same as in previous experiments (1). All the values given are corrected for self-absorption and are the averages of duplicates or triplicates; the individual values did not vary from the average by more than ±10 per cent.

TABLE I

Influence of Hydrogen Ion Concentration on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Concentration of added $CaCl_2$ in reaction mixture	Buffer	Buffer concentration in reaction mixture	pH of buffer	Gas mixture	pH at end of run	Counts per min. per mg. protein
	molal		*molal*				
1	0.0006	$NaHCO_3$	0.009		O_2	7.7	0.08
	0.0006	"	0.009		" + CO_2	7.1	0.2
	0.0006	"	0.005		"	6.8	0.4
	0.0006	"	0.005		" + CO_2	6.6	0.55
	0.0006	None			" + "	6.6	0.60
	0.0006	Phosphate	0.011	6.6	" + "	6.6	0.6
	0.0006	"	0.011	6.0	" + "	6.4	0.8
	0.0006	"	0.011	4.5	" + "	6.1	1.0
2	0.003	$NaHCO_3$	0.009		" + "	7.2	1.0
	0.003	Phosphate	0.011	6.6	" + "	6.5	1.35
	0.003	"	0.011	6.0	" + "	6.4	1.6
	0.003	"	0.011	5.7	" + "	6.15	2.3
	0.003	"	0.011	5.6	" + "	6.05	2.4
	0.003	Succinate	0.002	5.8	" + "	6.05	2.4
	0.003	Phosphate	0.011	4.5	" + "	5.7	1.45

Reaction mixtures incubated at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.03 M $CaCl_2$ in Experiment 1 and 0.15 M in Experiment 2; 0.1 ml. of buffer solution; 0.1 ml. of Ringer's solution without bicarbonate adjusted to pH 6.5 containing 1.6 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); and 0.3 ml. of undiluted homogenate. The pH values at the end of the run were after the addition of 4 ml. of water.

All the glassware and the saline solutions were sterilized before use by autoclaving for 20 minutes under 15 pounds steam pressure.

Results

Influence of Hydrogen Ion Concentration—The use of undiluted liver homogenate permitted only approximate pH control. The pH of the homogenate immediately after its preparation varied between 6.9 and 7.1. It became acid during incubation whether under 100 per cent oxygen or 95 per cent oxygen and 5 per cent carbon dioxide. The desired final pH was attained approximately by addition of buffers and incubation under either pure oxygen or 95 per cent oxygen plus 5 per cent carbon dioxide. It was found in separate trials that the pH found after incubation at 38° for 2 hours had been attained within half an hour.

The data in Table I show that when the whole homogenate was used the proteins gave the highest number of counts when the final pH of the reaction mixture was near 6.1. Whether bicarbonate, phosphate, fumarate, or succinate was used as a buffer, or whether the reaction was carried out under 100 per cent oxygen or 95 per cent oxygen plus 5 per cent carbon dioxide, the number of counts in the protein at any given pH was the same.

TABLE II

Influence of Hydrogen Ion Concentration on Incorporation of Labeled Lysine into Proteins of Sedimented Fraction of Guinea Pig Liver Homogenate

Buffer in which sedimented fraction was suspended	Initial pH of diluted sedimented fraction	Gas mixture	Final pH	Counts per min. per mg. protein
Succinate, 0.025 M	5.5	$O_2 + CO_2$	5.3	0.3
" 0.025 "	6.0	" + "	5.8	0.5
Ringer's solution without $NaHCO_3$	6.7	" + "	6.3	1.5
" " containing 0.23% $NaHCO_3$	7.4	" + "	7.2	3.9
Same	7.5	" + "	7.3	4.4
Ringer's solution containing 0.38% $NaHCO_3$	7.9	" + "	7.8	3.6
Same	8.5	" + "	8.1	3.4
"	8.85	"	8.8	3.0

Reaction mixtures incubated at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.2 M $CaCl_2$; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.5 containing 1.6 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); 0.4 ml. of the sedimented fraction diluted with half its volume of buffer solution, its initial pH adjusted, and incubated under the gas mixture indicated. The pH values at the end of the run were after the addition of 4 ml. of water.

The optimum pH with the sedimented fraction was at, or near, 7.3 (Table II). At the optimum pH nearly twice as many counts per mg. of protein were found in the proteins of the sedimented fraction as in those of the whole homogenate. Some of the counts obtained in the whole homogenate near or above pH 7 undoubtedly came from components of the sedimented fraction.

Influence of Calcium Concentration—Table III shows the influence of the concentration of calcium added to the reaction mixture. With the whole homogenate the counts in the protein were greater the greater the concentration of added calcium chloride up to a final concentration of 0.004 M; this was the case at hydrogen ion concentrations below and above the optimum. There is practically no further augmenting effect of calcium above 0.004 M; 0.01 M calcium chloride is not inhibitory; we have not explored the effect of higher concentrations.

TABLE III

Influence of Concentration of Calcium on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Homogenate fraction used	Concentration of added $CaCl_2$ in reaction mixture	Initial pH of phosphate buffer	pH at end of run	Counts per min. per mg. protein
		molal			
1	Whole, initial pH 6.9	None	4.5	6.1	0.9
	“ “ “ 6.9	0.001	4.5	6.1	1.8
	“ “ “ 6.9	0.002	4.5	6.1	2.0
	“ “ “ 6.9	0.003	4.5	6.0	2.3
	“ “ “ 6.9	0.004	4.5	6.0	2.5
	“ “ “ 6.9	0.005	4.5	6.0	2.6
	“ “ “ 6.9	0.01	4.5	6.0	2.6
2	“ “ “ 5.9	0.003	6.4	6.4	1.2
	“ “ “ 5.9	0.0006	6.4	6.4	0.4
	“ “ “ 5.9	0.003	6.4	6.1	1.7
	“ “ “ 5.9	0.0006	6.4	6.2	0.5
	“ “ “ 7.1	0.003	4.5	5.5	1.1
	“ “ “ 7.1	0.0006	4.5	5.6	0.5
3	Sedimented fraction, initial pH 7.8	0.004	No buffer used	7.6	4.0
	Same	None	“ “ “	7.5	3.3

Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the final concentration given; 0.1 ml. of 0.05 M phosphate buffer; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.0 containing 1.6 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); 0.3 ml. of undiluted homogenate or 0.4 ml. of sedimented fraction after dilution with an equal volume of Ringer's solution containing 0.38 per cent sodium bicarbonate. The pH values at the end of the run were after the addition of 4 ml. of water.

The calcium content of guinea pig liver reported in the literature ranges from 0.5 to 14.9 mg. per cent (15–17). The lowest concentration of added calcium which permitted a maximum number of counts in the protein was near 0.004 M or 16 mg. per cent. Changes in calcium content, and especially in the portion not combined with fatty acids, may, therefore, affect significantly the incorporation of lysine in the proteins of the liver.

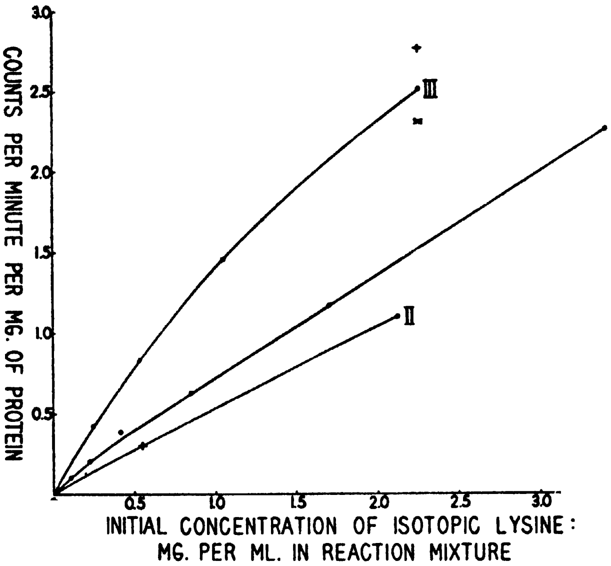

Fig. 1. Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours. Curve I, 1.01 ml. of the reaction mixtures containing 0.3 ml. of Ringer's solution without bicarbonate; 0.01 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate but with 0.06 M $CaCl_2$; 0.1 ml. of Ringer's solution, adjusted to pH 6.0, without bicarbonate, containing the radioactive L-lysine (18,300 counts per minute per mg.); 0.6 ml. of undiluted homogenate. After incubation, following the addition of 4 ml. of water, all mixtures were at pH 6.6. Curve II, 0.51 ml. containing 0.01 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate but with 0.05 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer at pH 6.0; 0.1 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate, containing the radioactive L-lysine (18,300 counts per minute per mg.); and (●) 0.3 ml. of undiluted homogenate, (+) 0.3 ml. of homogenate diluted 1:1 with Ringer's solution without bicarbonate. After incubation, following the addition of 4 ml. of water, all mixtures were at pH 6.4. Curve III, 0.51 ml. containing 0.01 ml. of Ringer's solution adjusted to pH 6.0, without bicarbonate but with 0.15 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer, pH 4.5; 0.1 ml. of Ringer's solution without bicarbonate, adjusted to pH 6.0, containing the radioactive L-lysine (9150 counts per minute per mg.); and (●) 0.3 ml. of undiluted homogenate, (+) 0.3 ml. of homogenate diluted 1:1 with Ringer's solution without bicarbonate, and (×) 0.3 ml. of homogenate diluted 1:2 with Ringer's solution without bicarbonate. After incubation, following the addition of 4 ml. of water, all mixtures were at pH 6.2.

Table III shows that the effect of added calcium was much less on the sedimented fraction than on the whole homogenate. Dounce and Beyer (18) give the concentration of calcium in rat liver nuclei as 2.3×10^{-4} gm. per 100 gm., wet weight, or approximately 6×10^{-5} M. On the assumption that the concentration in guinea pig liver nuclei is similar, and in view of the results with the whole homogenate, it seems unlikely that the lesser effect of added calcium on the sedimented fraction was because its calcium concentration was high initially.

Influence of Concentration of Lysine—The count per mg. of the proteins of the whole homogenate was close to a linear function of the initial concentration of labeled lysine (Fig. 1).

This result was obtained at pH 6.2, 6.4, and 6.6, and with 0.00049, 0.00059, and 0.0029 molal final concentrations of added $CaCl_2$. Provided the lysine, the $CaCl_2$ concentration, and the pH were kept constant, the subsequent count per mg. of the proteins was independent of the concentration of the homogenate.

TABLE IV

Effect of Total Amount of Labeled Lysine Available on Rate of Its Incorporation into Proteins of Sedimented Fraction of Guinea Pig Liver Homogenate

0.4 M $CaCl_2$	L-Lysine dihydrochloride solution, 16 mg. per ml.	Ringer's solution	Total volume of reaction mixture	Counts per min. per mg. protein
ml.	*ml.*	*ml.*	*ml.*	
0.01	0.1	0	0.51	3.3
0.02	0.2	0.4	1.02	6.7
0.04	0.4	1.2	2.04	10.5

The sedimented fraction (0.4 ml.) was used undiluted in this experiment. The $CaCl_2$ and lysine solutions were made up in Ringer's solutions without $NaHCO_3$ and adjusted to pH 6.0. The specific activity of the L-lysine dihydrochloride was 6100 counts per minute per mg. The Ringer's solution used for dilution contained 0.38 per cent $NaHCO_3$. The reaction mixtures were incubated under a mixture of 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours. The pH at the end of the run after the addition of 4 ml. of water was in every case 7.55.

When the sedimented fraction was used, the count per mg. of protein was dependent on the total amount of labeled lysine in the whole volume of the solution and not on its concentration (Table IV). Thus with a 4-fold increase in the volume of the reaction mixture with the initial labeled lysine concentration constant and the amount (but not the concentration) of sedimented fraction the same, the count per mg. of the protein was greater the greater the volume of the reaction mixture, *i.e.* the more labeled lysine made available to the sedimented fraction.

Progress of Reaction with Time in Whole Homogenate—The count per mg. of the protein attains, at 38°, its maximum in about 2 hours (Fig. 2). One factor which may be responsible for the rapid slowing down of the reaction is that a large fraction of the added lysine disappears. The major product (we have not yet identified it) is neither α-aminoadipic acid nor an immediate metabolic product of the latter.

The evidence for an additional factor which may be responsible for the cessation of the reaction was obtained in other experiments. After 2 hours incubation with labeled lysine, when the reaction had come nearly to a stop, the protein gave 2.4 counts per minute per mg. At this point the concentration of the isotope was increased to 4 times the initial value.

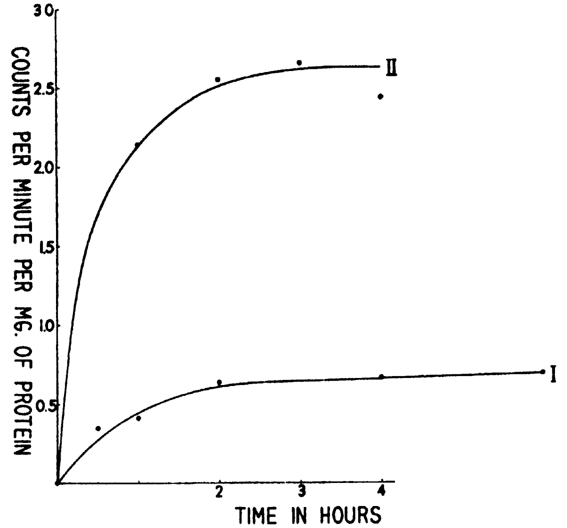

Fig. 2. Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38°. Curve I, 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.03 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer at pH 6.0; 0.1 ml. of Ringer's solution without bicarbonate adjusted to pH 6.0 containing 0.26 mg. of radioactive L-lysine dihydrochloride (12,200 counts per minute per mg.); 0.3 ml. of undiluted homogenate. After incubation, following addition of 4 ml. of water, all mixtures were at pH 6.4. Curve II, 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with 0.25 M $CaCl_2$; 0.1 ml. of 0.05 M phosphate buffer at pH 4.5; 0.1 ml. of Ringer's solution adjusted to pH 6.0, with bicarbonate, containing 2.13 mg. of L-lysine dihydrochloride (6100 counts per minute per mg.); 0.3 ml. of undiluted homogenate. After incubation, following addition of 4 ml. of water, all mixtures were at pH 6.2. The zero counts shown at zero time in Curves I and II are experimental determinations.

After another 2 hours incubation the count would be expected to be increased 4-fold in view of the almost linear relation between protein count and initial concentration of labeled lysine (Fig. 1), if the reaction system were in the same state after 2 hours as at the beginning. The count found, however, was only 3.7 per minute per mg. The increase was only 33 instead of the expected 400 per cent. Among the possible explanations, one is that most of the enzymatic activity is lost after 2 hours incubation; another is that the reaction is not an exchange of lysine in the protein but a combination to saturation at loci on the protein capable of combining with lysine, and that after 2 hours incubation with the initial addition of lysine it is nearly saturated. The almost linear relation in Fig. 1 argues against the latter interpretation, but it is not decisive.

Controls and Inhibitors—Boiling destroys most of the enzymatic activity in both the whole homogenate and in the sedimented fraction (Table V).

TABLE V

Effect of Boiling Homogenate Fraction and of D- *instead of* L-*Lysine on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate*

Experiment No.	Homogenate fraction used	Treatment of homogenate fraction before incubation	Lysine isomer used	Concentration of added $CaCl_2$ in reaction mixture	pH at end of run	Counts per min. per mg. protein
				molal		
1	Whole homogenate	None	L	0.0029	6.1	2.5
	" "	Boiled	"	0.0029	6.1	0.04
2	" "	None	"	0.00059	6.5	1.2
	" "	Boiled	"	0.00059	6.5	0.08
3	" "	None	"	0.0029	6.1	2.5
	" "	"	D	0.0029	6.1	0.6
4	" "	"	L	0.00059	6.5	1.2
	" "	"	D	0.00059	6.5	0.5
5	Sedimented fraction	"	L	0.0039	6.8	3.0
	" "	Boiled	"	0.0039	6.8	0.1
6	" "	None	"	0.0039	7.8	3.65
	" "	Boiled	"	0.0039	7.8	0.2

Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours; 0.51 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the molalities given; 0.1 ml. of 0.05 M phosphate at pH 4.5 in Experiments 1 and 3, and at pH 6.0 in Experiments 2 and 4; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.5 containing 1.6 mg. of L- or D-lysine dihydrochloride (12,200 counts per minute per mg. in Experiments 1 and 4, and 6100 counts per minute per mg. in the others); 0.3 ml. of undiluted homogenate in Experiments 1 to 4, 0.4 ml. of sedimented fraction after dilution with half its volume of Ringer's solution without bicarbonate and adjusted to pH 7.0 in Experiment 5, but with 0.38 per cent sodium bicarbonate and adjusted to pH 7.9 in Experiment 6. The pH values at the end of the run were after the addition of 4 ml. of water.

The procedure in this test was to transfer the whole homogenate or the sedimented fraction to the reaction vessel, immerse it in a boiling water bath for 15 minutes, cool, add the other components of the reaction system, stir them together thoroughly with a stirring rod, and then incubate. It will be noted that Table V ascribes some residual activity to the heated preparations. In the case of the whole homogenate the values given were not significantly above the background count;[1] they were significantly above the background in the boiled sedimented fractions.

[1] A sample is usually counted for 30 minutes. The background count in this interval is about 330. We do not consider a sample to have a count significantly above

The proteins gave some counts when D-lysine was used instead of L-lysine (Table V). The count was always much less than in the corresponding L experiment, and could be accounted for largely by the 7 to 15 per cent[2] of the L form in the D preparation used. From the results of feeding experiments (19) it would be expected that isotopic D-lysine would not be incorporated, if the sole reason for the indispensability of lysine is its incorporation into protein.

The experiments with boiled whole homogenate and sedimented fractions were also a check on the efficacy of the procedure used to wash out labeled lysine, which was not combined with protein. The zero counts at zero time shown in Fig. 1 provided evidence to the same effect. The experiments with labeled D-lysine served this purpose also to some extent.

Table VI summarizes experiments on the effects of anaerobiosis and of some oxidation inhibitors. Anaerobiosis does not inhibit the incorporation of lysine into the proteins of the whole homogenate; it does so, but only incompletely, in the sedimented fraction. 0.02 M fluoride inhibits almost completely in the whole homogenate and only slightly in the sedimented fraction. Arsenate, arsenite, azide, cyanide, and dinitrophenol (all in 0.001 M concentration) inhibit in the whole homogenate to varying degrees, all short of completion, and less so or not at all in the sedimented fraction. Similar results were obtained with the whole homogenate with the different inhibitors whether the concentrations of calcium and of hydrogen ion were optimum or suboptimum.

The fluoride inhibition in the whole homogenate was examined in more detail (Table VII). The results with 0.01 M fluoride show that fluoride was more effective at pH 6.2 than at 6.6. Rothschild (20) observed that fluoride inhibition of lipase was greater the lower the hydrogen ion concentration. Runnström and Sperber (21) found the same on the fermentation and respiration of yeast, and Warburg and Christian (22) on enolase.

Experiment 2 in Table VII was designed to test whether calcium and fluoride bear the same relation to the enzyme system in the whole homogenate as do magnesium and fluoride to enolase. Warburg and Christian showed that magnesium ion activates enolase and also increases the inhibition by fluoride. Their interpretation was that magnesium ion forms an enzymatically active complex with enolase protein and that magnesium, fluoride, and enolase protein form an inactive complex. In a quantitative study of the inhibition they found, in support of their interpretation, an equilibrium constant between the concentration of mag-

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the background unless its excess over the background is more than 3 times the standard deviation of the latter, in practice, 14 counts per minute or more.

[2] The optical rotation of the L preparation was $[\alpha]_D^{23} = +15.95°$, in water, $c = 5.46$; and of the D preparation, $[\alpha]_D^{23} = -13.5°$, in water, $c = 4.47$. The latter figure was the less reliable, as we had only a little of the D preparation.

TABLE VI

Effect of Oxidation Inhibitors on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Concentration of added $CaCl_2$ in reaction mixture	pH at end of run	Inhibitor, concentration in reaction mixture	Counts per min. per mg. protein	Degree of inhibition*
	molal				*per cent*
1	0.00059	6.5	None	1.2	
	0.00059	6.5	Anaerobiosis	1.25	
2	0.0049	6.6	None	0.81	
	0.0049	6.6	Anaerobiosis	0.77	5
	0.0049	6.6	Sodium arsenate (0.001 M)	0.59	27
	0.0049	6.6	" arsenite (0.001 ")	0.27	67
	0.0049	6.6	" azide (0.001 ")	0.46	43
	0.0049	6.6	Potassium citrate (0.005 ")	0.82	0
	0.0049	6.6	Sodium cyanide (0.001 ")	0.70	14
	0.0049	6.6	" fluoride (0.02 M)	0.04	95
3	0.0049	6.2	None	2.5	
	0.0049	6.2	Anaerobiosis	2.4	4
	0.0049	6.2	Sodium arsenate (0.001 M)	1.9	24
	0.0049	6.2	" arsenite (0.001 ")	1.2	52
	0.0049	6.2	" azide (0.001 ")	2.0	20
	0.0049	6.2	" fluoride (0.02 M)	0.1	96
	0.0049	6.2	Dinitrophenol (0.001 M)	1.8	28
4	0.0039	7.7	None	5.1	
	0.0039	7.7	Anaerobiosis	3.9	24
	0.0039	7.7	Sodium arsenate (0.001 M)	5.0	2
	0.0039	7.7	" arsenite (0.001 ")	5.1	0
	0.0039	7.7	" azide (0.001 ")	4.7	8
	0.0039	7.7	" fluoride (0.02 M)	4.4	14
	0.0039	7.7	Dinitrophenol (0.001 M)	5.4	0

Reaction mixtures incubated at 38° for 2 hours. The aerobic experiments were incubated under 95 per cent O_2 and 5 per cent CO_2; the anaerobic experiments under 95 per cent N_2 and 5 per cent CO_2. 0.52 ml. of the reaction mixtures containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the concentration given; 0.1 ml. of 0.1 M phosphate buffer at pH 6.0 in Experiments 1 and 2 and at pH 4.5 in Experiment 3; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.0 containing 1.6 mg. of L-lysine dihydrochloride (12,200 counts per minute per mg. in Experiment 1, and 6100 counts in Experiments 2 to 4); 0.3 ml. of undiluted whole homogenate in Experiments 1 to 3, and 0.4 ml. of sedimented fraction after dilution with half its volume of Ringer's solution containing 0.38 per cent sodium bicarbonate and its pH adjusted to 7.9 in Experiment 4; 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with the inhibitors 51 times the molalities given. The pH values at the end of the run were after the addition of 4 ml. of water.

* $\frac{\text{(Counts without inhibitor)} - \text{(counts with inhibitor)}}{\text{(Counts without inhibitor)}} \times 100.$

nesium and fluoride ions and the degree of inhibition. Najjar (23) found the same relations between magnesium, fluoride, and phosphoglucomutase activity, and gave the same interpretation. The data in Experiment 2 of Table VII show that the degree of inhibition by 0.01 M fluoride was the same in 0.005 M as in 0.001 M calcium. A calcium-fluoride-enzyme complex, which is inactive, as an explanation of the fluoride inhibition appears, therefore, to be excluded.

The following metabolites and salts neither accelerated nor inhibited the reaction in the whole homogenate, at either pH 6.6 with 0.0006 M added $CaCl_2$ or at pH 6.1 with 0.003 M added $CaCl_2$, nor did they relieve either the fluoride or arsenite inhibitions (the figures in parentheses are final concentrations in the reaction mixture): adenosine triphosphate (0.001 M), coenzyme I (20 mg. per cent), potassium citrate (0.005 M), creatine (200 mg. per cent), cysteine (0.005 M), cytochrome (65 mg. per cent), fumarate (0.005 M), D-glucose (100 mg. per cent), glucose-1-phosphate (75 mg. per cent), glutathione (300 mg. per cent), glycerophosphate (0.005 M), hexose diphosphate (180 mg. per cent), α-ketoglutarate (0.005 to 0.01 M), oxalacetate (0.005 M, slightly inhibitory), 2-phosphoenolypyruvate (0.005 M), 3-phosphoglycerate (0.005 M), pyridoxal phosphate (50 to 250

TABLE VII

Inhibiting Effect of Fluoride on Incorporation of Labeled Lysine into Proteins of Guinea Pig Liver Homogenate

Experiment No.	Concentration of added $CaCl_2$ in reaction mixture	NaF concentration in reaction mixture	pH at end of run	Counts per min. per mg. protein	Degree of inhibition
	molal	*molal*			*per cent*
1	0.00059	None	6.6	1.21	
	0.00059	0.02	6.6	0.04	97
	0.00059	0.01	6.6	0.64	47
	0.00059	0.0025	6.6	0.94	22
	0.00059	0.001	6.6	1.22	0
2	0.0049	None	6.2	1.99	
	0.0049	0.02	6.2	0.12	94
	0.0049	0.01	6.2	0.69	65
	0.001	None	6.2	0.83	
	0.001	0.02	6.2	0.08	96
	0.001	0.01	6.2	0.30	64

Reaction mixtures incubated under 95 per cent O_2 and 5 per cent CO_2 at 38° for 2 hours; 0.52 ml. containing 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with $CaCl_2$ 51 times the molality given; 0.01 ml. of Ringer's solution without bicarbonate at pH 6.0 with sodium fluoride 51 times the molality given; 0.1 ml. of 0.05 M phosphate buffer at pH 6.0 in Experiment 1 and at pH 4.5 in Experiment 2; 0.1 ml. of Ringer's solution without bicarbonate at pH 6.0 containing 1.6 mg. of L-lysine dihydrochloride (12,200 counts per minute per mg. in Experiment 1, and 6100 counts per minute per mg. in Experiment 2); 0.3 ml. of undiluted homogenate. The pH values at the end of the run were after the addition of 4 ml. of water.

mg. per cent), pyridoxine (1 to 10 mg. per cent), pyruvate (0.005 M), $CoCl_2$ (0.00005 to 0.0005 M), $CuCl_2$ (0.0005 to 0.005 M), $MgCl_2$ (0.0005 to 0.005 M), $MgSO_4$ (0.00005 to 0.0005 M), $MnCl_2$ (0.0005 to 0.005 M), $ZnCl_2$ (0.0005 to 0.005 M). Replacement of all the sodium by potassium salts and *vice versa* in the Ringer's solution similarly neither accelerated nor inhibited the reaction.

We investigated whether the counts in the proteins of the whole homogenate were incorporated as lysine or in some other form. 300 mg. of the protein, giving a total of 360 counts per minute, were hydrolyzed by refluxing for 20 hours with 20 per cent hydrochloric acid. The lysine was isolated as the picrate by Block's modification (24) of the Kossel procedure: this is a specific and standard method for the isolation of lysine.

The lysine picrate isolated gave, after one recrystallization from water, the following elementary analysis.

$C_{12}H_{17}O_9N_5$. Calculated. C 38.36, H 4.57, N 18.66
375.30 Found. " 38.29, " 4.47, " 18.46

The picrate gave 20 counts per minute per mg. of lysine; further recrystallization did not change this count. According to Block and Bolling (24) the lysine content of liver proteins is 6.3 per cent. On the basis that all the counts in the protein came from the lysine, its 360 counts correspond to a "calculated" count of its lysine of 19 counts per minute per mg., which is the value found in the lysine isolated, within the accuracy of the measurement.

Another portion of the protein was hydrolyzed in the same manner and then precipitated with phosphotungstic acid. The precipitate gave 330 counts per minute; the protein before hydrolysis gave 360 counts; the filtrate from the phosphotungstic precipitate gave no counts.

On the basis of the foregoing data the lysine incorporated accounted for all the radioactivity of the protein.

A measure of the extent to which a labeled amino acid is incorporated into a protein is the fraction of that amino acid isolated from the protein found to be carrying the label. In the foregoing instance the lysine isolated gave 20 counts per minute per mg. The lysine added to the homogenate gave 9150 counts per minute per mg. 20/9150 × 100 or 0.22 per cent of the lysine in the protein was, therefore, labeled. Nearly all of this incorporation occurred in the 1st hour of incubation (Fig. 2).

DISCUSSION

Studies we have now in hand with labeled leucine and glycine caution against generalizing from the findings on labeled lysine to the incorporation of other amino acids into homogenate proteins. Leucine and glycine, under the best conditions we have found so far for lysine, are incorporated far more slowly than lysine; this is the case both with the whole homogenate and the sedimented fractions of guinea pig and rat liver.

SUMMARY

1. Two sets of conditions were found in guinea pig liver homogenate in which L-lysine (labeled with C^{14} in the ϵ position) is incorporated into the proteins in relatively large amounts. In one case the enzyme system was the whole homogenate, in the other the precipitate obtained by centrifuging the 15-fold diluted homogenate at 2500*g*.

2. Characteristics of the reaction with the whole homogenate are as follows: its optimum pH is approximately 6.1; the presence of calcium is obligatory, the concentration of the latter for maximum activation being approximately 0.004 M; 0.01 M does not inhibit; the reaction proceeds as well in nitrogen as in oxygen; it is inhibited nearly completely by 0.02 M fluoride, 50 per cent by 0.001 M arsenite, and somewhat less by 0.001 M arsenate, azide, cyanide, and dinitrophenol; the concentration of L-lysine incorporated into the proteins is approximately a linear function of the lysine concentration and is independent of the concentration of the homogenate.

3. Characteristics which differentiate the reaction with the sedimented fractions from that with the whole homogenate are as follows: its optimum pH is near to 7.3; the reaction is accelerated only a little by calcium, and the presence of calcium is not obligatory; it proceeds more slowly under nitrogen than under oxygen, but is not completely inhibited by anaerobiosis; it is inhibited slightly by 0.02 M fluoride, and practically not at all by 0.001 M arsenate, arsenite, azide, or dinitrophenol; the concentration of lysine incorporated is a function of the amount of lysine in the total volume of the solution in which the sedimented fraction is suspended.

4. The rate of incorporation of labeled lysine into the proteins of the whole homogenate is approximately the same as that *in vivo;* after 1 hour's incubation at 38° about 0.15 per cent of the lysine in the proteins is labeled. This rate is 60 times faster than any hitherto reported in whole homogenates for other amino acids.

5. The rate of incorporation of labeled lysine into the proteins of the sedimented fraction, under the most favorable conditions for the latter, is several times that in the whole homogenate under its most favorable conditions.

6. All the radioactivity found in the proteins of the whole homogenate was accounted for as labeled lysine.

The microanalyses were carried out by Mr. G. Swinehart. The authors were assisted by A. A. Dvorsky, H. E. Jeffrey, M. Keighley, and A. Tollestrup.

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A PEPTIDE FRACTION IN LIVER*

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We reported in a preliminary communication (1) the isolation of a peptide fraction from guinea pig liver. The following points of interest appeared at once: many different amino acids were obtained on hydrolysis; the peptide fraction contained most of the indispensable amino acids, which indicated that it probably is important in protein metabolism; when guinea pig liver homogenate was incubated with C^{14}-labeled glycine, leucine, or lysine, these were rapidly incorporated into this peptide fraction,[1] which is further evidence that it is metabolically active; the peptide fraction had not been described hitherto; a fraction containing one or more large peptides can be separated from so complex a mixture as liver homogenate by starch chromatography.

We have not yet established whether the peptide fraction obtained by chromatographic isolation is a mixture which chromatographs as a unit or whether it consists of a single peptide. The following evidence favors the latter alternative. The fraction was precipitated by picric, flavianic, or trichloroacetic acids; after removal of the precipitating acid the fraction behaved the same chromatographically as it did before. Its amino acid composition was not demonstrably different before or after precipitation with picric acid nor before or after precipitation with ether from aqueous ethanol. The same chromatographic peptide fraction was found in the liver of fish, beef, guinea pig, hog, horse, lamb, and rat, and in guinea pig blood, diaphragm, heart, kidney, and spleen; this fraction from such widely different sources contained the same amino acids and their relative proportions were similar. Furthermore we have found this fraction to be one of the major products arising in the peptic hydrolysis of bovine serum albumin, bovine γ-globulin, casein, fibrin, insulin, and ovalbumin. (The details of this finding will be presented in a later communication.) Data are presented below on the peptide fraction isolated from Witte's peptone

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* This work is a part of that done under contract with and joint sponsorship of the Office of Naval Research, United States Navy Department, and the United States Atomic Energy Commission. The C^{14} used in this investigation to label amino acids was supplied by the Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

[1] The details of these findings will be reported later.

(which is derived from a peptic hydrolysate of fibrin). Its chemical behavior and amino acid composition were similar to those of the same chromatographic fraction obtained from liver. The foregoing evidence indicates that the chromatographic fraction is an operational entity; tentatively it will be considered as such pending further evidence from chemical and metabolic studies now in hand.

We have designated this fraction as Peptide A. There are other metabolically active peptides in liver which we are now engaged in identifying.

In view of the fact that this peptide fraction is an operational entity in a number of respects, the present communication describes methods of isolation, some of its physical and chemical properties, the amount in the liver of a number of different animals and in some guinea pig tissues, and its amino acid composition as obtained by different methods and from different sources.

Procedure

The tissues used were those of adult guinea pigs and of white rats procured from several commercial and laboratory sources, of fish from a cannery,[2] and of abattoir beef, hog, horse, and lamb.

The guinea pigs and rats were killed by stunning and bled. The guinea pig tissues and rat livers were chilled and worked up immediately. The livers of other animals were frozen with solid CO_2 a few minutes after removal from the animals and kept frozen until they were worked up.

The tissue was first disintegrated in a Waring blendor and then homogenized in the apparatus of Potter and Elvehjem (2). The homogenate was diluted with 25 times its weight of water, the pH adjusted to 5.0, and then boiled for 10 minutes. The coagulated protein was removed by filtration and reextracted three times by boiling with water. The original filtrate and the washings were combined and concentrated to dryness by distillation *in vacuo* at a bath temperature of 50°.

The residue was then chromatographed on starch by the method of Stein and Moore (3). Amino acids, peptides, and other substances are eluted from a starch column by a continuous flow of a selected solvent. Different substances appear in the eluent, separately or in groups in a definite order. The eluent is collected in fractions. When these are analyzed with ninhydrin reagent, the intensity of color is proportional to the amount of amino nitrogen in the fractions. This intensity of color (conveniently expressed in terms of amino nitrogen on the basis of an amino acid standard), when plotted consecutively against volume of effluent which has emerged, gives a "spectrum" consisting of bands of varying width and height (Fig. 4). The total color in the band tells the amount of substance.

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[2] For which we wish to thank Dr. E. Geiger, Van Camp Laboratories, Terminal Island, California.

Chromatography of Tissue Extracts: Peptide A Fraction—The following is an example of the quantities of tissue, starch,[3] and solvents used. The dried residue of the non-protein filtrate from 25 gm. of liver was dissolved in 1.5 ml. of N HCl and 30 ml. of a solvent mixture consisting of 1 part of 0.1 N HCl, 2 parts of *n*-propanol, and 1 part of *n*-butanol (4). This solution was transferred to the top of a column containing 1 kilo of starch, and forced into it with slight air pressure; the sides of the column were washed down with three small portions of the HCl-propanol-butanol mixture, each washing being forced into the top of the column before the next was added. The same solvent mixture was used for elution.

The dimensions of the starch column were 80 × 300 mm. The rate of flow of eluent was 75 to 90 ml. per hour. This rate was obtained by adjusting the pressure to 10 to 15 cm. of Hg, applied by either air or nitrogen.

0.5 ml. aliquots of the fractions were analyzed by the modification by Moore and Stein (5) of the ninhydrin method.

Chromatography of Hydrolysates—For the determination of the amino acid composition of the Peptide A fraction, 2 to 8 mg. were hydrolyzed by refluxing with 20 per cent HCl at a bath temperature of 150° for 20 hours. After removal of the excess acid the dry residue was chromatographed on a 7 to 10 × 300 mm. starch column, containing 21 to 25 gm. of starch, with the HCl-propanol-butanol solvent mixture as eluent. The rate of flow was adjusted to 1 to 2.5 ml. per hour; three fractions were collected per hour. After about 200 such fractions the following amino acids (if present) have been eluted, and in this order: leucine, isoleucine, phenylalanine, tryptophan, methionine, tyrosine, valine, proline, glutamic acid, alanine, threonine, aspartic acid, serine, and glycine.

At this point, it is possible by changing the eluent to a mixture consisting of 2 parts of *n*-propanol and 1 part of 0.5 N HCl to accelerate the emergence of ammonia, the bases, and cystine (4). In our experience the subsequent chromatogram was often ragged. This procedure was useful nevertheless; it indicated the presence of the bases and it permitted computation of the total amino nitrogen (reacting with ninhydrin) after acid hydrolysis. Other methods were used to establish the identity of the ammonia and amino acids emerging after the change in eluting solvent.

Chromatographic Isolation of Peptide A Fraction—The Peptide A fraction is eluted from the starch column by the HCl-propanol-butanol solvent mixture a short time before the first free amino acid, which is leucine (Fig.

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[3] Two batches of white potato starch were used: one was obtained from the Amend Drug and Chemical Company, Inc., New York; the other was manufactured by the Idaho Potato Starch Company, Blackfoot, Idaho.

1). When the peptide fraction is rechromatographed, it emerges again in the same place (Fig. 2).

Distribution of Peptide A Fraction—Table I gives figures on the amounts of Peptide A fraction found in the livers of a number of animals and in a number of guinea pig tissues. The non-protein extracts were prepared and chromatographed as described above. The figures in the second column were obtained by converting the total color given with the ninhydrin reagent in the fractions comprising the Peptide A band into amino nitrogen on the basis of the leucine standards. The figures in the last column were obtained from those in the second by applying the factor 6.5 γ of amino nitrogen = 1 mg. of peptide. This factor was given by preparations of the peptide fraction isolated by a number of different pro-

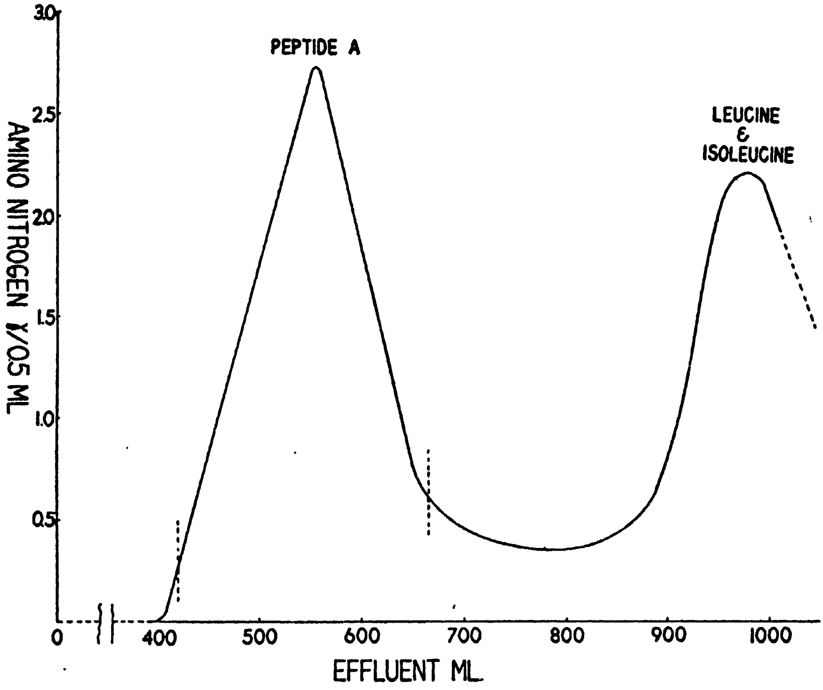

FIG. 1. Chromatographic separation of the Peptide A fraction from the non-protein filtrate of guinea pig liver. The first portion of the chromatogram of the non-protein filtrate from 23 gm. of guinea pig liver. Solvent, 1:2:1, 0.1 N HCl-*n*-propanol-*n*-butanol; column, 1000 gm. of starch, diameter 80 mm., height 300 mm. The eluent was collected in 32 ml. fractions; 0.5 ml. aliquots were analyzed. The concentration of amino nitrogen calculated from the ninhydrin color referred to that given by the leucine standards. One-half of the combined fractions between the vertical dotted lines was rechromatographed (see Fig. 2).

cedures described in the next section. The figures for mg. per cent of the peptide fraction are maximum values and probably are somewhat too high; a small admixture of material of low molecular weight giving color with the ninhydrin reagent would result in a great magnification of the amount of peptide estimated by applying the factor 6.5 γ = 1 mg. of peptide. This reservation does not, in view of all the evidence, invalidate the use of the ninhydrin values as an indication of the relative amounts of the peptide fraction in different tissues. On this basis the data in Table I indicate that liver contains large amounts of the Peptide A fraction; spleen is almost as rich, and there is little or none in striated skeletal muscle.

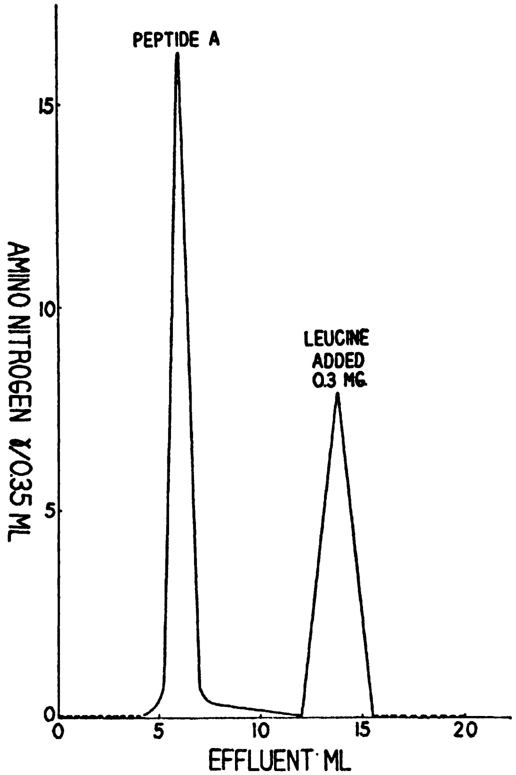

FIG. 2. The Peptide A fraction isolated as shown in Fig. 1 rechromatographed with 0.3 mg. of leucine added. Solvent, 1:2:1, 0.1 N HCl-*n*-propanol-*n*-butanol; column 21 gm. of starch, diameter 7 mm., height 275 mm. The eluent was collected in 0.35 ml. fractions; a whole fraction was analyzed.

Physical and Chemical Properties and Chemical Isolation of Peptide A Fraction—The properties described in this section are those of the Peptide A fraction from guinea pig liver, rat liver, and from Witte's peptone. We found no difference between them. We shall in this section for convenience refer to this fraction as if it were a single peptide; as stated above it may consist of a number of peptides of similar composition and behavior. The peptide is soluble in water; its hydrochloride is soluble in 80 per cent ethanol and in absolute methanol; it is precipitated from solution in the two latter solvents by ether. As was to be expected from its amino acid composition, it gives a picrate and a flavianate. It is precipitated by high concentrations (10 to 20 per cent) of trichloroacetic acid; dilute solutions of the peptide are less completely precipitated than concentrated solutions. It gives a purple biuret test, and positive tests with Millon's and Hopkins-Cole reagents.

TABLE I

Peptide A Fraction in Some Animal Tissues

Sources	Amino nitrogen, γ per 100 gm. tissue, wet weight	Mg. per 100 gm. tissue, wet weight*
Albacore liver	2600	400
Beef liver	3600	550
Guinea pig liver	3500	530
Hog liver	3350	510
Horse "	1200	180
Lamb "	1170	180
Rat "	2700	410
Guinea pig blood	115	18
" " diaphragm	Trace	Trace
" " heart	470	72
" " kidney	250	38
" " spleen	2100	320
" " striated muscle of abdominal wall	0	0

* The values in this column were obtained by applying the factor 6.5 γ of amino nitrogen (ninhydrin) = 1 mg. of Peptide A.

The foregoing physical and chemical properties provided several methods for isolating the peptide, either from starch and other materials in the HCl-propanol-butanol mixture in which it was eluted from the starch column, or, when Witte's peptone was the source, from other materials in the peptone mixture. The preparations obtained by the following isolation procedures are not to be construed as pure.

The chromatographic Peptide A fraction of 25 gm. of either guinea pig or rat liver was dried in a current of air. The residue was stirred in 10 ml. of 95 per cent ethanol, and water was then added dropwise until practically all the material was dissolved. This was centrifuged and to the clear supernatant solution ether was added dropwise until a heavy precipitate appeared. The precipitate was submitted to the same procedure twice more, then washed with ether, and dried. The dried material, assayed with the ninhydrin reagent, gave 6.5 γ of amino nitrogen per mg. It gave before hydrolysis the same chromatogram as the original Peptide A fraction. Fig. 5 is the chromatogram of the preparation from rat liver after it was hydrolyzed.

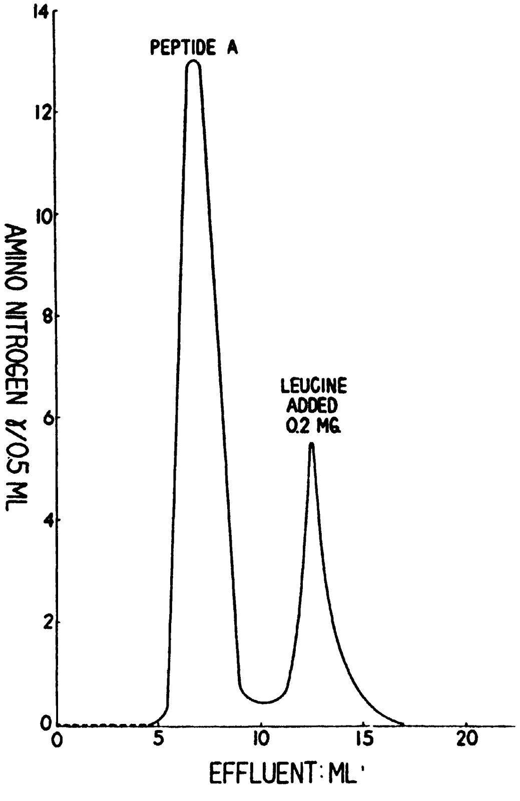

FIG. 3. Chromatogram of the Peptide A fraction isolated via the picrate from Witte's peptone as described in the text. 8 mg. chromatographed with 0.2 mg. of added leucine. Solvent, 1:2:1, 0.1 N HCl-n-propanol-n-butanol; column, 25 gm. of starch, diameter 9 mm., height 290 mm. The eluent was collected in 0.6 ml. fractions; 0.5 ml. aliquots were analyzed.

Peptide A was isolated as the picrate from the starch column eluate of the non-protein fraction of guinea pig liver and from Witte's peptone which had not been chromatographed. After the eluent fraction was evaporated to dryness, the subsequent procedure was the same in the isolation from both sources. To the aqueous solution of the eluent fraction or of Witte's peptone a saturated aqueous solution of picric acid was added until maxi-

TABLE II

Comparison of Amino Acid Composition of Peptide A Fraction from Different Sources

Source	Treatment of Peptide A before hydrolysis	Amino nitrogen in aliquot				Ratio of amino nitrogen in other bands to that in leucine + isoleucine bands				
		Before hydrolysis	After hydrolysis	Ratio, after hydrolysis to before hydrolysis	In leucine + isoleucine bands*	Methionine + tyrosine†	Glutamic acid + alanine	Threonine + aspartic acid	Serine	Glycine
		γ	γ		γ					
Albacore liver	None				64.4	0.60	1.38	1.18		
Beef liver	"	30.2	403	13.3	53.7	0.76	1.39	1.08	0.41	0.59
Guinea pig liver	"	95.9	1288	13.4	173.8	0.86	1.48	1.19	0.41	0.64
" " "	"				59.5	0.64	1.29	0.92	0.31	0.59
" " "	"	30.1	413	13.7	54.9	0.60	1.37	1.16	0.44	0.78
" " "	Isolated as picrate‡	22.9	363	15.8	50.4	0.62	1.18	1.03	0.44	0.79
Hog liver	None	28.4	454	15.9	64.2	0.65	1.36	1.02	0.41	0.61
Horse liver	"	43.2	655	15.1	97.3	0.57	1.32	1.05	0.42	0.67
Lamb "	"	38.9	552	14.1	76.4	0.83	1.24	1.02	0.36	0.71
Rat "	"	38.6	529	14.7	67.9	0.60	1.43	0.94	0.41	0.73
" "	"	32.1	461	14.3	67.5	0.74	1.35	0.98	0.38	0.67
" "	Isolated from solution in ethanol§	13.0	191	14.6	25.4	0.79	1.31	1.08	0.44	0.76
" "	Same	13.0	206	15.8	24.8	0.62	1.41	1.18	0.43	0.78
Witte's peptone	Isolated as picrate‖	13.0	179	13.7	23.1	0.73	1.52	1.48	0.43	0.78
Guinea pig blood	None				79.4	0.75		1.07	0.37	0.66
" " "	"				18.2	0.58	1.29		0.36	0.58
" " kidney	"				52.5	0.52	1.38	1.08	0.47	0.68
" " spleen	"				44.9	0.52	1.25	1.01		

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* Phenylalanine was omitted from the designation of the leucine-isoleucine band because only questionable amounts were found by colorimetric, ultraviolet, and chromatographic methods.

† The valine was omitted from the designation of the methionine-tyrosine band because the values found by colorimetric determination of the methionine and colorimetric and ultraviolet determination of the tyrosine accounted for practically all the amino nitrogen in the band.

‡ Prepared as follows: the Peptide A fraction precipitated with picric acid, the picric acid removed from the precipitate with ether, and the residue hydrolyzed.

§ Prepared as follows: the Peptide A fraction evaporated to dryness; residue dissolved in 80 per cent ethanol from which it was precipitated with ether. Solution in 80 per cent ethanol and precipitation with ether repeated twice more, the residue dried, and 2 mg. hydrolyzed and the hydrolysate chromatographed. The amino nitrogen value before hydrolysis was obtained by direct assay of the isolated preparation.

‖ Aqueous solution of Witte's peptone precipitated with picric acid; the picric acid removed from the precipitate with ether; the residue chromatographed; the Peptide A fraction evaporated to dryness in air and dissolved in 80 per cent ethanol, from which it was precipitated with ether; and 2 mg. of the dried precipitate hydrolyzed and chromatographed. The amino nitrogen value before hydrolysis was obtained by direct assay on the isolated preparation.

mum precipitation had occurred. After standing overnight in the refrigerator the suspension was centrifuged, the precipitate dissolved in a minimum amount of HCl and the picric acid extracted with ether. Ethanol was then added to a final concentration of 80 per cent and the peptide precipitated with ether as described above. Data on these preparations are given in Figs. 3 and 6 and in Table II.

The simplest isolation from Witte's peptone was as follows. 200 mg., dissolved in 50 ml. of water, were precipitated with 10 per cent (final concentration) of trichloroacetic acid. The trichloroacetic acid was removed from the precipitate with ether, and the residue was dried with absolute ethanol and ether. The yield was 14 mg. The chromatogram before hydrolysis was the same as that of all other preparations of the Peptide A fraction; 6.5 γ of amino nitrogen (by ninhydrin) per mg. were obtained.

A preparation isolated from Witte's peptone via the flavianate gave the same amino nitrogen value and the same chromatogram. 2.0 gm. of Witte's peptone were suspended in 70 ml. of absolute methanol; 0.7 ml. of concentrated HCl was added and after thorough shaking the mixture was centrifuged. The precipitate was suspended in a fresh 70 ml. portion of methanol and 0.7 ml. of HCl and the extraction was repeated. In all, five such extractions were made, the residue from one extraction being used for the next. Twice the volume of absolute ether was added to the combined supernatant solutions. The precipitate was separated by centrifuging and then dried. It weighed 620 mg. 200 mg. of the dried precipitate were dissolved in 2 ml. of water, and to the solution were added 100 mg. of flavianic acid in 2 ml. of water. The gummy, orange precipitate was separated from the yellow supernatant liquid by centrifuging. 0.2 N H_2SO_4 was added to the precipitate until it was strongly acid and the flavianic acid was extracted with amyl alcohol. The clear residual solution was treated with $Ba(OH)_2$ to remove the sulfate and with CO_2 to remove the excess barium and was then evaporated to dryness. The yield was 30 mg.

Amino Acid Composition of Peptide A Fraction—The amino acid composition of the Peptide A fraction was determined by starch chromatography after acid hydrolysis as described above. The results are given in Figs. 4, 5, and 6 and in Table II. The material hydrolyzed was either that in the central region of the Peptide A band of the non-protein filtrate of a tissue or the preparations isolated as described in the previous section. Moore and Stein (5) give the positions of the different amino acids in the chromatogram obtained by elution first with HCl-propanol-butanol and then with propanol-HCl. We have used a number of tests to check these positions and have confirmed Moore and Stein completely. C^{14}-labeled leucine and glycine (and lysine, not shown in Figs. 4, 5, and 6) were added

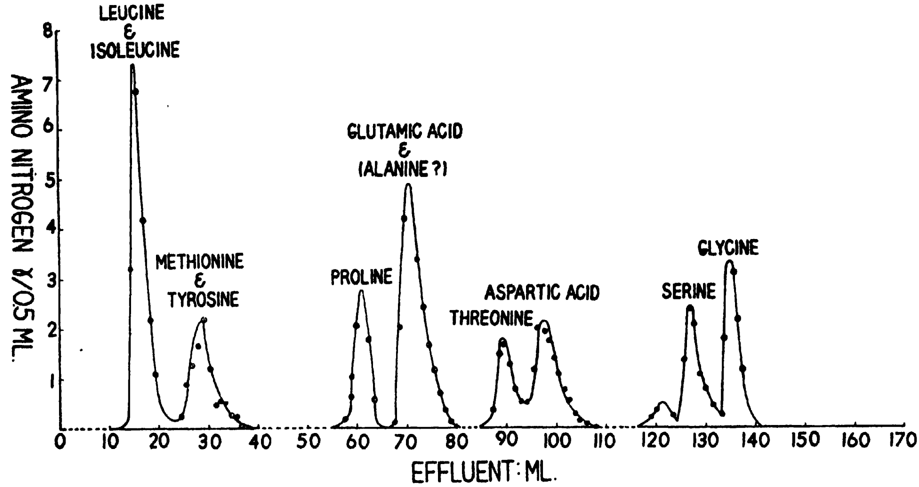

Fig. 4. Chromatogram of the hydrolysate of 20 per cent of the Peptide A fraction of guinea pig liver shown in Fig. 1. Solvent, 1:2:1, 0.1 N HCl-*n*-propanol-*n*-butanol; column, 25 gm. of starch, diameter 9 mm., height 298 mm. The eluent was collected in 0.55 ml. fractions; 0.5 ml. aliquots were analyzed. The portion of the chromatogram after the glycine band is not shown.

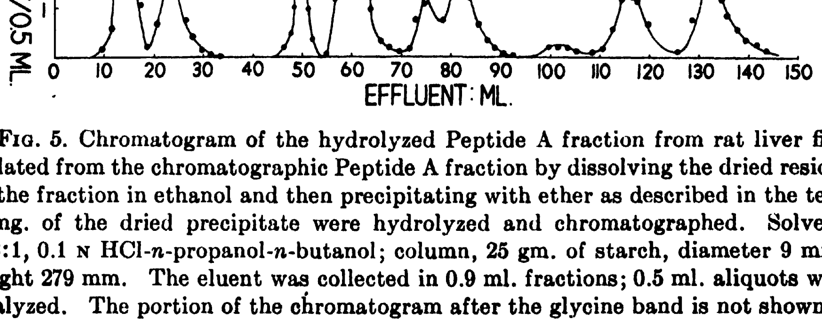

Fig. 5. Chromatogram of the hydrolyzed Peptide A fraction from rat liver first isolated from the chromatographic Peptide A fraction by dissolving the dried residue of the fraction in ethanol and then precipitating with ether as described in the text. 2 mg. of the dried precipitate were hydrolyzed and chromatographed. Solvent, 1:2:1, 0.1 N HCl-*n*-propanol-*n*-butanol; column, 25 gm. of starch, diameter 9 mm., height 279 mm. The eluent was collected in 0.9 ml. fractions; 0.5 ml. aliquots were analyzed. The portion of the chromatogram after the glycine band is not shown.

to aliquots of a hydrolysate and the coinciding radioactivity and ninhydrin peaks found in the fractions of eluent. Leucine, tyrosine, proline, threonine, aspartic acid, serine, and glycine were located by a loading procedure. A relatively large amount of one amino acid was added to an aliquot of a hydrolysate; this loaded aliquot and another to which no addition was made were chromatographed at the same time on two separate columns. The chromatograms of the two portions of the hydrolysate were the same except for the heightened peak in one, corresponding to the amino acid added.

Proline gives a yellow color with the ninhydrin reagent. The presence in the peptide fraction (but not the location in the hydrolysis chromatogram) of tryptophan (6), methionine (7), and tyrosine (8), and arginine and histidine (9) was determined colorimetrically. The ultraviolet spectrum of the unhydrolyzed Peptide A fraction was measured and was practically completely accounted for by the content of tryptophan and tyrosine as found colorimetrically.

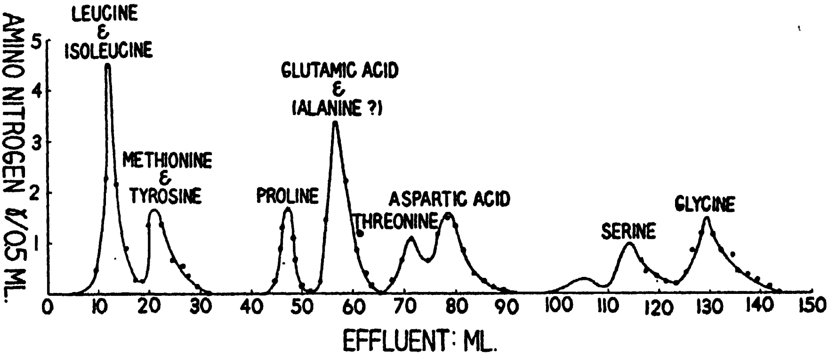

FIG. 6. Chromatogram of the Peptide A fraction from Witte's peptone, isolated via the picrate as described in the text. 2 mg. were hydrolyzed and then chromatographed. Solvent, 1:2:1, 0.1 N HCl-n-propanol-n-butanol; column, 25 gm. of starch, diameter 8 mm., height 295 mm. The eluent was collected in 0.9 ml. fractions; 0.5 ml. aliquots were analyzed. The portion of the chromatogram after the glycine band is not shown.

The presence of lysine was established also by the use of *lysineless Neurospora*, mutant 4545 (10).[4] Unhydrolyzed Peptide A fraction will not support growth of this mutant unless a small amount of free lysine is added to the culture medium. Hydrolyzed Peptide A fraction requires no such seeding. Evidently the minimum growth afforded by a trace of free lysine provides hydrolytic enzymes which liberate lysine from the peptide (or peptides) for further growth.

The presence of isoleucine in the leucine band was established by chromatography on starch according to Stein and Moore (3), with 1:1:0.284 n-butanol-benzyl alcohol-water as the eluting solvent. This solvent mixture separates phenylalanine, leucine, and isoleucine. The presence of leucine was established by using C^{14}-labeled leucine as a tracer, and that of isoleucine by the loading procedure described above.

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[4] We wish to thank Mr. E. Windsor of this laboratory for carrying out the lysine assays with the *Neurospora* mutant.

Filter paper chromatography with phenol and *s*-collidine (11) of the glutamic acid + alanine fractions gave several spots; among them were those which could be assigned to glutamic acid and alanine, but the picture was complicated by the presence of spots given by substances which were not identified.

The presence in the Peptide A fraction from every source listed in Table II of the following amino acids has been established by the methods referred to above: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, and tyrosine. Chromatography on starch by elution with the *n*-butanol-benzyl alcohol-water solvent, which separates phenylalanine, leucine, and isoleucine (3), indicated that phenylalanine or some other amino acid which emerges before leucine may be present. If phenylalanine is present there is very little. To establish this point it will be necessary to prepare and highly purify much larger amounts of the peptide or peptides in the fraction than we have at present. The same considerations apply to cystine, valine, and other amino acids which may be present, as for example the substance giving the low rounded peak between aspartic acid and serine shown in Figures 4, 5, and 6.

Figs. 4, 5, and 6 are chromatograms of hydrolyzed Peptide A fraction from three different sources, guinea pig liver, rat liver, and Witte's peptone. They are clearly the same qualitatively. Similar chromatograms were given by hydrolyzed Peptide A from all the sources listed in Table I. These chromatograms indicated the same qualitative amino acid composition of the Peptide A fractions from different sources, isolated by different methods.

Table II summarizes data which indicate that the quantitative amino acid composition of Peptide A from different sources, isolated by different methods, was similar. Table II gives values of amino nitrogen before and after acid hydrolysis. Except those indicated in the foot-notes the amino nitrogen values before hydrolysis were obtained from the chromatogram of the Peptide A fraction. All the values after hydrolysis were obtained by adding the ninhydrin values of all the bands obtained by elution first with the HCl-propanol-butanol solvent followed by the propanol-HCl mixture. All the values of the ratio after hydrolysis to before hydrolysis fall within the range 13.3 to 15.9; the average is 14.5.

The concordance of the values of this ratio further supports treatment of the chromatographic Peptide A fraction as an operational entity. The value of 14.5 of the ratio of free amino nitrogen after hydrolysis to before hydrolysis is within the range found for proteins. Thus Henriques and Gjaldbaek (12) by formol titration found values for dried egg white, casein, edestine, gelatin, and Witte's peptone of 14.6, 8.8, 24.4, 22.7, and 5.6 respectively. We may conclude, therefore, that most of the Peptide A fraction chromogenic with the ninhydrin reagent consists of polypeptide material of large molecular weight.

Table II gives the ratios of the amino nitrogen in the main bands to that of leucine + isoleucine. The figures in Table II show that the Peptide A fractions of different origin appear to have similar amino acid composition. This statement applies not only to the comparisons in Table II but also to their content of lysine. The *Neurospora* assays indicated that they all contain 9 to 10 per cent of lysine. This value probably is low, as arginine and glutamic acid, both of which are present in the peptide, inhibit this mutant. In every case it was necessary to add a trace of free lysine to initiate growth, and there was the same lag of 24 to 48 hours before rapid growth began.

Ratios of the amino acid groups were compared in Table II because we do not consider the Peptide A fractions to have been pure enough to warrant comparison of the content of individual amino acids expressed as absolute amounts. It is premature, we feel, to stress the differences in the ratio of the same amino acid group. Stein and Moore (3) in replicate analyses of purified proteins show divergencies of 10 per cent between extreme values for one amino acid. Each analysis in Table II was done on a specimen of Peptide A fraction obtained from a different animal. The notable finding is that the fraction obtained from widely different sources was so similar with respect to all five amino acid groups compared to leucine plus isoleucine and with respect to the ratio of amino nitrogen before and after hydrolysis. *A priori* no such similarity was to be expected.

DISCUSSION

The following considerations bear on the questions of the purity of the Peptide A fraction from any one source and the identity or difference of this fraction obtained from different sources. Contamination by free amino acids is excluded because these emerge from the starch column (with the eluting solvent employed) after the Peptide A fraction. The Peptide A fraction is precipitated by 10 per cent trichloroacetic acid; small peptides are thereby excluded as contaminants. Further evidence in the same direction is that a number of dipeptides and glutathione emerge after leucine, whereas the Peptide A fraction emerges before leucine. The Peptide A fraction is precipitated by picric and flavianic acids; contamination by peptides not containing basic amino acids is thereby excluded.

The amino acid composition of the original chromatographic fraction was not demonstrably different from that of the fraction after precipitation either with picric acid or with ether from aqueous ethanol. Also the ratio of free amino nitrogen (with ninhydrin reagent) before and after hydrolysis was the same for the three materials. This ratio is of the order to be expected of a peptide containing the fifteen (or more) amino acids present in the fraction. Therefore, if the Peptide A fraction is a mixture, it is a mixture of similar large peptides.

SUMMARY

1. The chromatographic isolation of a peptide fraction in liver, designated Peptide A, is described. Several chemical methods of isolating it are described. This fraction contains one or more large peptides.

2. The presence of the following amino acids in the Peptide A fraction has been established: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tryptophan, and tyrosine. Additional amino acids may be present.

3. The Peptide A fraction has been isolated from the livers of albacore, beef, guinea pig, hog, horse, lamb, and rat, from guinea pig blood, heart, kidney, and spleen, and from Witte's peptone. Liver and spleen are the richest sources; there is much less in blood, heart, and kidney and very little or none in diaphragm and striated muscle of the abdominal wall.

4. The Peptide A fraction isolated from different sources contains all of the above amino acids. Their proportions are not demonstrably different within the limits of precision of the analytical methods used.

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METABOLIC FUNCTIONS OF BIOTIN*

I. THE RÔLE OF BIOTIN IN BICARBONATE UTILIZATION BY LACTOBACILLUS ARABINOSUS STUDIED WITH C^{14}

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A metabolic relationship between biotin and aspartic acid has been demonstrated in many living forms. Burk, Winzler, and du Vigneaud (1) observed that either biotin or aspartate was capable of stimulating the fermentation and nitrogen assimilation of biotin-deficient yeast (*Saccharomyces cerevisiae*) in media containing ammonia. Independently Koser, Wright, and Dorfman (2) found that aspartate partially replaced biotin for *Torula cremoris*.

In 1947, Stokes *et al.* (3) presented evidence that biotin, in some manner, promotes the synthesis of aspartate by several bacterial species. A study of the mechanism by which biotin affects the synthesis of aspartate was therefore undertaken. It was found (4) that oxalacetate could partially replace aspartate in promoting growth of biotin-deficient *Lactobacillus arabinosus*. Since Lyman *et al.* (5) had shown that CO_2 stimulates aspartate synthesis by lactobacilli, it seemed likely that biotin was exerting its influence on aspartate synthesis by way of the Wood-Werkman (6) condensation of pyruvate and CO_2 to yield oxalacetate. Growth experiments did indeed demonstrate that bicarbonate greatly stimulated growth of *L. arabinosus* in aspartate-free media if biotin were present and that bicarbonate elicited no growth response in low biotin media (4). Using their technique of *inhibition analysis*, Shive and Rogers (7) independently reached the conclusion that biotin participated in CO_2 fixation into oxalacetate and α-ketoglutarate. About the same time Lichstein and Umbreit (8) demonstrated that biotin restored the ability of aged *Escherichia coli* cells to decarboxylate oxalacetate.

To obtain further information on the function of biotin, studies were made of the uptake of C^{14}-labeled bicarbonate by normal and biotin-deficient cells of *L. arabinosus*.

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Methods and Materials

The organism *Lactobacillus arabinosus* 17-5 was grown on the basal medium described in a previous communication (9). Four modifications of this medium, the biotin and aspartic acid composition of which was that suggested by Stokes *et al.* (3), were used in the experiments reported below. The low biotin medium contained 0.05 mμgm. of biotin and 0.2 mg. of DL-aspartic acid per ml.; the high biotin medium contained 2.0 mμgm. of biotin per ml. and no aspartic acid; the "oleic acid" medium contained 10 γ of oleic acid, 0.5 mg. of Tween 40, and 0.2 mg. of DL-aspartic acid per ml., and the biotin-free, aspartic acid-free medium contained neither biotin nor aspartic acid. The optical density of bacterial cultures was measured in the Evelyn colorimeter with standard size tubes at 660 mμ and with water as a blank. The bacteria were grown in volumes of 25 to 100 ml. in Erlenmeyer flasks filled to about 50 per cent of capacity. After sterilization the flasks were aseptically inoculated with 0.5 to 1.0 ml. of a washed saline suspension of an 18 hour culture diluted to 85 to 90 per cent transmission. The size of inoculum is of some importance, since the organism may show a prolonged lag period on the high biotin medium if too light an inoculum is used. The techniques described above will give cultures showing 50 to 60 per cent transmission in about 18 hours with the first three media mentioned above. In the first experiments reported in this communication the organisms grown on low and high biotin media were made up to equal optical density for transfer to the Warburg vessel by diluting the more dense culture with the same type of medium. Later, when the inhibitory effect of aspartic acid became known, the cells were removed from the growth medium by centrifuging and resuspended in biotin-free, aspartate-free medium. They were centrifuged again and made up to equal optical density in biotin-free, aspartate-free medium. About 2½ hours elapsed from the time centrifugation began until the cells were transferred to the Warburg flasks.

C^{14} fixation Experiments were carried out at 37° in Warburg vessels with air as the gas phase. Bicarbonate, containing the isotope, and other materials were added from a side arm at zero time. The final concentration of added bicarbonate was 0.0033 M unless otherwise specified. After incubation at 37° for the desired time, dilute sulfuric acid (sufficient to bring the suspension to about pH 2) was dumped from a second side arm to stop metabolic reactions. The flask contents were transferred quantitatively to centrifuge tubes and pieces of solid CO_2 large enough to sink to the bottom of the tubes were added. After 10 minutes of "washing" in this manner (control vessels were found devoid of C^{14}) the tubes were centrifuged and the supernatant fluid decanted. The cells were autoclaved for

1 hour in the presence of 1 N HCl and the resulting solutions evaporated to dryness under a vacuum.

All samples were counted on flat bottomed shallow aluminum cups of 1 cm. radius. Samples were transferred to the cups as slurries in alcohol and were dried under an infra-red lamp. Counts were made with an end window Geiger counter tube. Mica windows of 2 to 3 mg. per sq. cm. were used. The counts were corrected for background and for self-absorption according to the calculations of Yankwich *et al.* (10, 11). Sufficient counts were made to establish an accuracy of about ±5 per cent in all samples except those in which the count was not appreciably greater than the background. The data are reported as counts per second for the total sample. Volumes of the cell suspensions are given for each experiment.

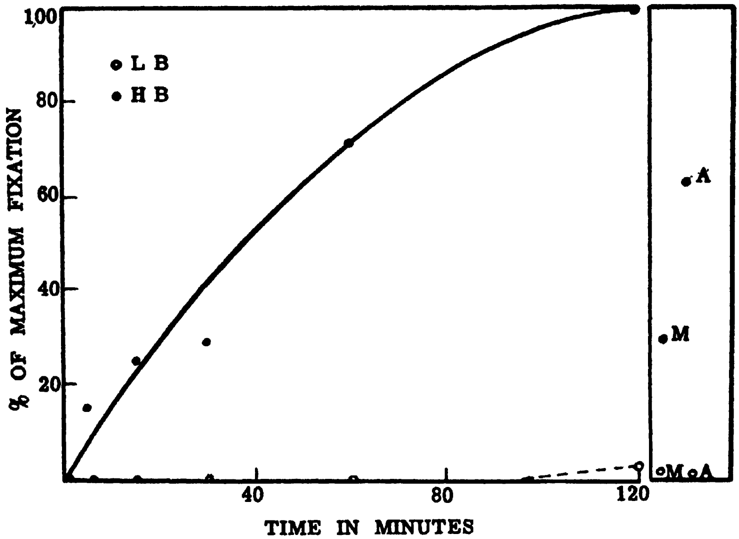


FIG. 1. Fixation of C^{14}-labeled bicarbonate by *L. arabinosus* in low biotin (*LB*) and high biotin (*HB*) media. 3 ml. of cell suspension (54 per cent transmission) per flask. Maximum fixation, 3.04 counts per second. The column at the right indicates the relative amounts of C^{14} fixed into the medium (*M*) and the cellular aspartic acid (*A*) by the two types of cells.

Control vessels containing high biotin cells which had been heated in a boiling water bath for 5 minutes were included in each experiment. In no case was any detectable quantity of C^{14} fixed by these inactivated controls.

Results

The rates of bicarbonate uptake by cells of *L. arabinosus* in low biotin and high biotin media are shown in Fig. 1. High biotin cells rapidly fixed C^{14} from the bicarbonate of the medium and assimilated it as acid-stable cell constituents, while the low biotin cells fixed no detectable amount during the 1st hour of the experiment. A trace, but an analytically significant amount, was fixed by the low biotin cells after 2 hours. This incorporation of C^{14} into the bacterial cell is not a net uptake of CO_2. There was a continuous net production of CO_2 by the cells during the experiment. At 120 minutes the cell-free medium from the high biotin cells contained an appreciable quantity of fixed C^{14}, while only an insignificant amount was found in the medium from the low biotin cells. The relative quantities fixed in the media are indicated in Fig. 1 (*M*). In this and later experiments in which media were fractionated, the "medium" would include all constituents which were extracted from the cells at pH 2. An appreciable part of this fixed C^{14} may therefore actually have risen from soluble cellular constituents.

25 mg. of natural aspartic acid were added as a carrier to each of the 2 hour cell hydrolysates and the aspartic acid was isolated and purified as the copper salt. The relative activity of the isolated copper aspartates is shown in the column at the right of Fig. 1. While a quantitative separation of the aspartate was not achieved, calculations indicate that most if not all of the activity of the cells was present in the aspartic acid.

In the experiment summarized in Fig. 1 two variables, biotin and aspartic acid, were studied simultaneously. To determine whether aspartate alone influenced bicarbonate fixation a similar experiment was performed and biotin was added to one series of cells in low biotin medium containing aspartate. In addition L-aspartate was added to one flask containing cells in high biotin medium. The results are summarized in Fig. 2. Again rapid uptake of C^{14} from the medium occurred with high biotin cells, while those grown and incubated in the low biotin medium containing aspartate fixed almost none. In this experiment the rate of C^{14} fixation by the high biotin cells was remarkably linear during 1 hour. Additions of 0.065 mμgm. of biotin per ml. of media at zero time did not appreciably increase the uptake of $C^{14}O_2$ by the deficient cells. However, the addition of 1 mg. of L-aspartate per ml. to the high biotin medium decreased the fixation almost to zero. While this is 10 times the concentration of aspartate in the low biotin medium, it seemed possible that the presence of aspartate in that medium might be preventing added biotin from exhibiting any $C^{14}O_2$-fixing response. A third experiment was therefore conducted in which the cells were grown on the high and low biotin media as usual, but, after centrifuging, both types of cells were suspended in sterile biotin-free, aspartate-free medium. The rates of $C^{14}O_2$ fixation are shown in Fig. 3. Cells grown in the high biotin medium fixed $C^{14}O_2$ fairly rapidly during the first 30 minutes of the experiment, but the rate dropped off during the second half hour. The addition of 5 mμgm. of biotin per ml. did not significantly influence the rate of $C^{14}O_2$ fixation during the first half hour but was effective in main-

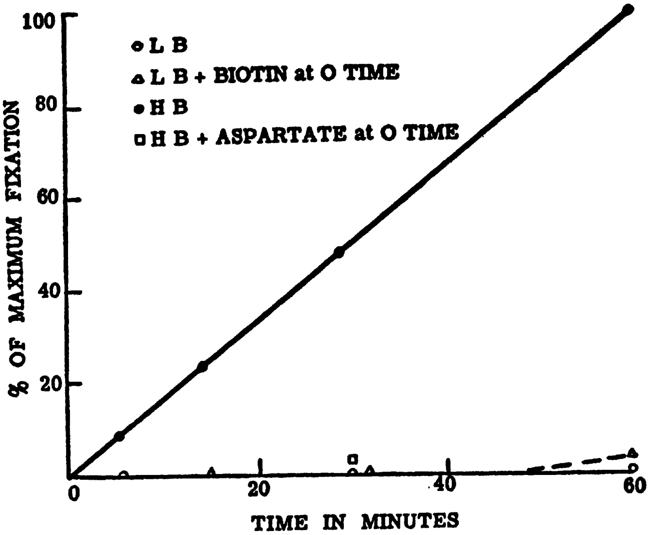


FIG. 2. Fixation of C^{14}-labeled bicarbonate by *L. arabinosus* in low biotin and high biotin media. 4 ml. of cell suspension (72 per cent transmission) per flask. Maximum fixation, 13.3 counts per second. Additions where indicated; biotin, 0.065 mμgm. per ml.; L-aspartate, 1 mg. per ml.

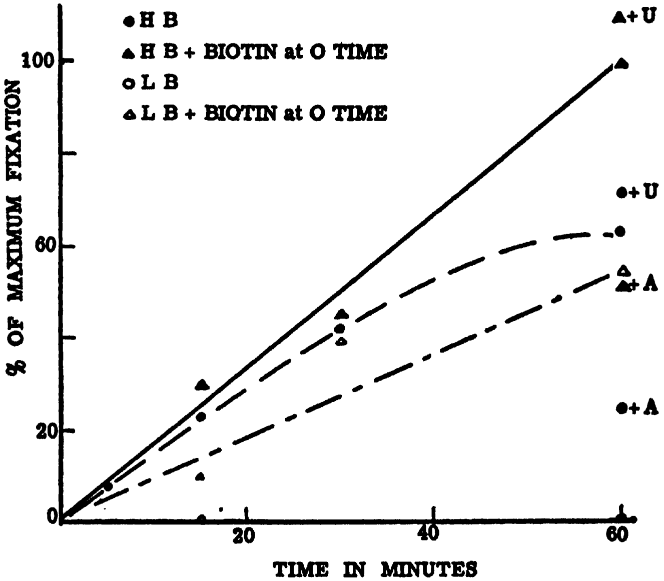


FIG. 3. Fixation of C^{14}-labeled bicarbonate by high biotin and low biotin cells of *L. arabinosus* in biotin-free, aspartate-free medium. Bicarbonate (final concentration 3.3×10^{-3} M) and, when added, biotin (5 mμgm. per ml.), DL-aspartate (4.7×10^{-4} M) (flasks labeled *A*), and γ-(3,4-ureylenecyclohexyl)-butyric acid (5 γ per ml.) (flasks labeled *U*) were dumped from the side arms at zero time. 2 ml. cell suspension (60 per cent transmission) per flask. Maximum fixation, 7.90 counts per second.

taining the rate of fixation during the second half hour. Cells grown on the low biotin medium were unable to fix $C^{14}O_2$ but did so when biotin was added to the aspartate-free medium. The addition of 63 γ of DL-aspartic acid per ml. of cell suspension decreased the rate of $C^{14}O_2$ incorporation by more than 50 per cent. The effect of varying concentrations of aspartate was then studied in a separate experiment, with the results shown in Fig. 4. Since 50 γ of L-aspartic acid per ml. inhibited C^{14} uptake as much as did 100 γ of DL-aspartic acid, the inhibition appears to result only from the L isomer.

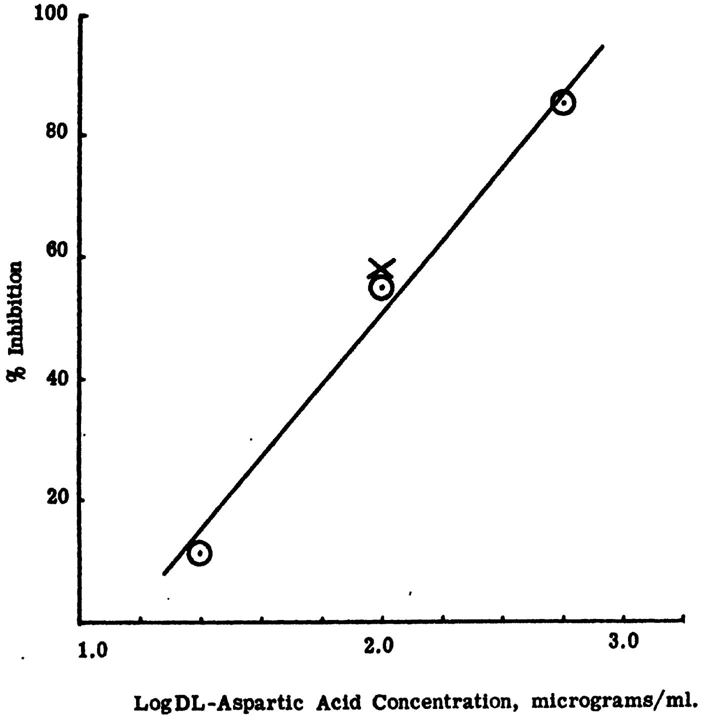


FIG. 4. Inhibition by aspartate of $C^{14}O_2$ fixation by *L. arabinosus*. High biotin cells (1.5 ml. of suspension; 17.5 per cent transmission) in biotin-free, aspartate-free medium. Final volume 2.0 ml.; incubated 60 minutes. Control fixation, 3.49 counts per second. ⊙ = DL-aspartic acid; × = one-half as much L-aspartic acid.

It should be emphasized that in all of the experiments described above the low biotin cells were grown in the presence of sufficient biotin to satisfy the "oleic acid function" (9, 12, 13). Therefore, it appears that the oleic acid function of biotin does not involve the fixation of carbon dioxide.

The results of the experiment described in Fig. 1 indicated that most of the radioactive carbon taken up was fixed into cellular aspartic acid. To make a more detailed examination of the distribution of C^{14}, low and high biotin cells were incubated in 100 ml. quantities with C^{14}-containing bicarbonate for 15 minutes. The washed cells were hydrolyzed by autoclaving 12 hours in 8 N H_2SO_4; 25 mg. of L-aspartic acid were added to each sample and the barium salts of aspartic and glutamic acids were separated by alcohol precipitation. The aspartic acid was separated from the crude barium salts as the copper salt and recrystallized. The percentage of total radioactivity recovered in the copper aspartate corresponded roughly to the percentage recovery of the latter, indicating that aspartic acid was the main if not the sole C^{14}-containing compound.

Adenine and guanine were isolated from the hydrolysate by the procedure of Levene (14) after adding 20 mg. of guanine hydrochloride and 20 mg. of adenine sulfate as carriers. Adenine separated as the silver salt, isolated, and purified as the picrate contained a trace, but statistically insignificant amount, of radioactivity. Guanine, separated as the silver salt and isolated as the free base was devoid of activity. It should be recalled that the basal media supply the purine bases adenine, guanine, and xanthine.

In these large scale experiments, the total counts of C^{14} fixed in the media were equivalent to 30 per cent of the amount retained by the cells. The media were continuously extracted with ether for 12 hours. After evaporation of the ether the residues from both high and low biotin cells were found to contain no detectable quantity of radioactivity. Almost all of the activity was found in the alcohol-precipitated barium salt fraction containing the dicarboxylic amino acids. Since the media were not hydrolyzed, the activity must have resided in the free amino acids. The cells either lose an appreciable amount of aspartic acid by diffusion into the medium or else the extraction at pH 2 removes labeled aspartate from the cells.

Four samples of pure copper aspartate isolated from cells exposed to C^{14}-containing bicarbonate were treated with chloramine-T according to the procedure of Cohen (15). In standardizing runs, recoveries of 96, 102, and 96 per cent of the theoretical CO_2 were obtained, assuming that both carboxyl groups were converted to CO_2. Cupric ion did not inhibit decarboxylation of standard samples. Recoveries of the radioactivity in the CO_2 liberated by chloramine-T ranged from 66 to 100 per cent of that originally present in the aspartic acid, but no appreciable amount of activity was found in any of the residues following the decarboxylation. Apparently part of the $C^{14}O_2$ had been lost from the $Ba(OH)_2$ trap in some of the experiments.

Action of Biotin Analogues—The results of the experiment summarized in Fig. 3 demonstrated that γ-(3,4-ureylenecyclohexyl)-butyric acid did not inhibit carbon dioxide fixation when added to cells which had been grown in the presence of sufficient biotin to satisfy the "aspartate function" (9, 3).

Thus the compound appeared not to antagonize the function of biotin already incorporated into the bacterial cell protoplasm. To examine more closely the locus of action of antibiotin compounds the experiment described in Table I was performed. Since γ-(3,4-ureylenecyclohexyl)-butyric acid is a comparatively weak antimetabolite (16), the more active compounds homobiotin and homobiotin sulfone (17) were employed. As can be seen from Table I, both of these biotin analogues strongly inhibited the fixation of $C^{14}O_2$ when added simultaneously with biotin to cells grown in the absence of biotin. They exerted only a slight inhibition of C^{14} fixation by cells which had been grown in high biotin medium. These results indicate that biotin analogues do not inhibit appreciably the function of biotin which has already been incorporated into cellular components, and that they do inhibit the formation of the biotin-containing tissue component which is concerned with CO_2 fixation into aspartic acid. Axelrod, Purvis, and Hofmann (18) have recently reported that biotin antagonists inhibit the biotin stimulation of yeast fermentation (1) only when added 30 minutes prior to the addition of biotin.

TABLE I

Effect of Biotin Analogues on $C^{14}O_2$ Assimilation by L. arabinosus

Type of cells	Biotin added	Inhibitor added	Net counts per sec.			Per cent inhibition
			10 min.	30 min.	60 min.	
	mμgm. per ml.					
No biotin	None	None	0.00		0.07	
	5	"	0.40	1.09	2.61	
	5	Homobiotin sulfone, 20 γ per ml.			0.58	78
	5	Homobiotin, 40 γ per ml.			0.51	80
High biotin	None	None	1.15	2.09	3.49	
	"	Homobiotin sulfone, 20 γ per ml.			3.27	6
	"	Homobiotin, 40 γ per ml.			3.02	13

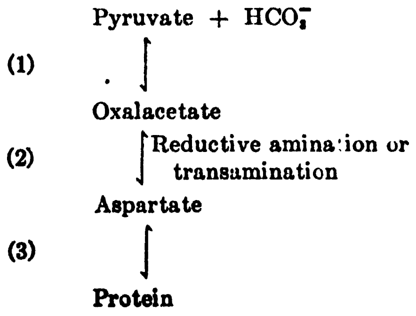
No biotin cells were grown in the presence of aspartic and oleic acids with Tween 40; turbidity at 22 hours ≅ 48 per cent transmission in the Evelyn colorimeter, Filter 660. High biotin cells were grown in the presence of 2 mμgm. of biotin per ml.; no aspartic or oleic acid added; turbidity at 22 hours ≅ 54 per cent transmission. After centrifuging and washing, both cell types were taken up in the basal medium and diluted to 17.5 per cent transmission, 1.5 ml. per flask. Biotin, 0.01 m.eq. of $NaHCO_3$ containing C^{14}, and the inhibitors were dumped from the side arms at zero time. Final volume 2.0 ml.

DISCUSSION

The striking differences in CO_2-fixing ability between cells grown in high biotin and low biotin media strongly indicate that biotin plays a compulsory rôle in the metabolic fixation of CO_2. In the present work aspartic acid was found to contain almost all of the C^{14} assimilated from the bicarbonate of the medium into the bacterial cell. This must not be construed to mean that the function of biotin is limited to fixing bicarbonate into the carbon skeleton of this single amino acid, for the present experiments were designed to supply the bacterial cells with all other required metabolites. If the incorporation of CO_2 into other cellular constituents is inhibited by the respective compound in the same manner in which added aspartate inhibits CO_2 fixation into cellular aspartic acid (Fig. 4), then, in these experiments, one would not expect C^{14} fixation into those other cellular constituents. Evidence for the concept that biotin is involved in the fixation of CO_2 into several tissue components in rats is presented in Paper II (19).

Studies of the function of biotin at the enzymatic level have not yet disclosed the exact mechanism by which this vitamin influences cellular metabolism. Ochoa *et al.* (20) found that biotin-deficient turkeys were specifically deficient in the enzyme which catalyzes the condensation of pyruvate and CO_2 in the presence of reduced triphosphopyridine nucleotide to produce malate. However, purified preparations of the enzyme were reported not to contain significant quantities of biotin (20). Ochoa therefore postulated that biotin might function as a catalyst in the synthesis of the enzyme rather than as a structural component of the enzyme. While the present work offers no test of this hypothesis, there is no particular evidence of a lag phase in the rate of CO_2 fixation when biotin is added to cells grown in low biotin medium (Fig. 3; Table I), such as one might expect if it were acting indirectly. Since malate does not give a growth response under conditions in which oxalacetate does (4), it appears not to be an intermediate in the CO_2 fixation process.

The inhibition by aspartate of CO_2 uptake may offer a clue to the mechanism of aspartate synthesis. In higher animals, tissue amino acids are constantly being exchanged for those being carried by the blood stream. If aspartate synthesis were occurring in bacteria by processes unrelated to protein synthesis, one would expect some fixation of $C^{14}O_2$ into aspartate, even though aspartate were present in the medium.



It seems possible that the energy-coupling mechanism which incorporates aspartate into protein (Reaction 3) may actually be the driving force which permits the endergonic coupling of CO_2 and pyruvate (Reaction 1).

We are indebted to Dr. M. W. Goldberg, Dr. S. H. Rubin, and Dr. E. Sevringhaus of Hoffmann-La Roche, Inc., for gifts of biotin, homobiotin, and homobiotin sulfone. Dr. R. O. Roblin of the American Cyanamid Company generously supplied the sample of γ-(3,4-ureylenecyclohexyl)-butyric acid. We wish to thank Dr. Charles Heidelberger for making several of the measurements of radioactivity.

SUMMARY

Cells of *Lactobacillus arabinosus* grown in the presence of 2 mμgm. of biotin per ml. fixed C^{14} from the bicarbonate of the medium into cellular aspartic acid. When grown in media containing only 0.05 mμgm., or less, of biotin per ml., the cells were almost completely unable to fix CO_2. The fixation of CO_2 into cellular aspartate was strongly inhibited by addition of aspartate to the medium.

Biotin analogues having antimetabolite properties inhibited the fixation of CO_2 when added simultaneously with biotin to low biotin cells. They had little influence on the CO_2-fixing capacity of cells grown in the presence of biotin.

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METABOLIC FUNCTIONS OF BIOTIN*

II. THE FIXATION OF CARBON DIOXIDE BY NORMAL AND BIOTIN-DEFICIENT RATS

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Since the early work of Wood and Werkman (1), which demonstrated the assimilation of CO_2 by heterotrophic non-photosynthetic bacteria, considerable interest has been shown in the function of carbon dioxide as a metabolite. Ruben and Kamen (2) provided evidence for the fixation of CO_2 by rat liver slices in the presence of pyruvate. This was extended by Evans and Slotin (3) with the use of $C^{11}O_2$ to show that the reaction involved the condensation of CO_2 with pyruvate to form oxalacetate. The synthesis of urea by liver slices and homogenates has also been shown to involve CO_2 fixation (4, 5) which occurs in the conversion of ornithine to citrulline (6). The intact animal can introduce CO_2 into liver and muscle glycogen (7) and into several amino acids of liver protein; namely, arginine, glutamic acid, and aspartic acid (8). It has recently been shown that the pigeon can fix $C^{14}O_2$ into uric acid (9).

The first suggestion that CO_2 transfer might involve a metabolic function of biotin was made by Burk and Winzler (10). The relationship between aspartic acid and biotin has been discussed in Paper I (11). Recently it has been demonstrated that *Lactobacillus arabinosus* grown on a biotin-deficient medium cannot utilize CO_2 for growth (12), but is able to assimilate either aspartate or oxalacetate for good growth (12, 13). The present study was undertaken to investigate the comparative abilities of the "normal" and biotin-deficient rat to utilize CO_2.

EXPERIMENTAL

Male albino rats of the Sprague-Dawley strain were used in all experiments and were raised from weaning on the following rations. The *control* ration contained alcohol-extracted casein 22, sucrose 68.5, Salts IV (14) 4.5, and corn oil 5 parts. Vitamins were added in the following amounts

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per 100 gm. of ration: thiamine 30 γ, riboflavin 60 γ, niacin 200 γ, pyridoxine 30 γ, calcium pantothenate 300 γ, *p*-aminobenzoic acid 500 γ, biotin 1 γ, folic acid 2.5 γ, inositol 10 mg., and choline 20 mg. 2 drops of halibut liver oil were administered weekly. The *low biotin ration* consisted of casein 20, commercial dried, raw, egg white 11, sucrose 60.5, Salts IV 4.5, corn oil 5.0 parts, and vitamins as in the control ration with the omission of biotin. The *low biotin, low fat ration* contained casein 20.5, dried raw egg white 11.0, sucrose 64.0, Salts IV 4.5 parts, and vitamins as in the control ration with the omission of biotin.

After 7 weeks, the animals fed the low biotin and low fat, low biotin rations reached a weight plateau and exhibited the characteristic skin symptoms of a biotin deficiency. 2 weeks later, one rat was taken from each of the three groups to be used for a preliminary experiment. Each rat was given, at 1 hour intervals, twelve equal doses of a solution of $NaHC^{14}O_3$ by intraperitoneal injection. The total amount given to the control rat was equivalent to 1.10 mg. of original $BaCO_3$ having a specific activity of 3 per cent C^{14}, and the amounts given to the other two rats were calculated in proportion to their metabolically effective weights by the factor $(\text{weight})^{0.73}$ (15, 16).

At the end of the 12 hour injection period, each animal was sacrificed by decapitation and the liver quickly removed and minced in a Waring blendor with 10 per cent trichloroacetic acid. The kidney, spleen, pancreas, heart, testes, and small intestine were combined as one fraction (hereafter referred to as the viscera fraction) and similarly treated. Both trichloroacetic acid precipitates were centrifuged and washed with 5 per cent trichloroacetic acid. The lipide fraction was removed from each by three extractions with boiling 3:1 alcohol-ether. The residue was then extracted with 5 per cent hot trichloroacetic acid at 90° for 20 minutes to remove the nucleic acid fraction (17).

The skeleton with its attached muscle was boiled for 30 minutes in distilled water, and the muscle stripped from the bones. The muscle tissue was minced in a Waring blendor and extracted with alcohol-ether in the same manner as the liver and viscera were, to remove the lipides.

The protein residue from each of the three fractions was hydrolyzed by refluxing with 8 N H_2SO_4 (10 ml. per gm.) for 24 hours, and the sulfate removed by treatment with $Ba(OH)_2$. The filtrate and washings from the $BaSO_4$ were combined, concentrated, and tyrosine removed by crystallization at its isoelectric point. The filtrate was adjusted to pH 3 to 4, and arginine isolated as its monoflavianate. After removal of excess flavianic acid and concentration of the solution, hot, saturated $Ba(OH)_2$ was added, to pH 10. The solution was filtered and 4 volumes of ethanol added. After refrigeration, the barium salts of glutamic and aspartic acids were centrifuged, washed, dissolved in a minimum of water, filtered, and reprecipitated. The barium was removed and the glutamic acid precipitated as its hydrochloride and aspartic acid obtained as its copper salt.

The skeleton was extracted with 3:1 alcohol-ether, dried, and ground to a fine powder. This was suspended in water, and concentrated hydrochloric acid (2 ml. per gm.) added slowly with shaking. The CO_2 evolved was collected in a solution of sodium hydroxide and precipitated by the addition of an excess of 10 per cent barium chloride solution. The barium carbonate was centrifuged, washed with CO_2-free water until the washings were neutral to litmus, and dried *in vacuo*. Additions of acetone were used to prevent the $BaCO_3$ precipitates from "creeping" during manipulations.

The bone residue in dilute hydrochloric acid solution was extracted with ether (4 ml. per gm. of original bone) for 24 hours, and the ether extraction repeated twice according to the method of Dickens (18) for the isolation of bone citric acid. The ether extracts were combined, made alkaline by the addition of $NaHCO_3$ solution, and the aqueous alkaline layer removed. The ether layer was washed with $NaHCO_3$ solution and the washings combined with the original alkaline layer. This was made acid with HCl and boiled to drive off CO_2. The solution was treated with norit, concentrated *in vacuo* to a small volume, and $CaCl_2$ solution was added. Calcium citrate was precipitated by making the solution faintly alkaline with ammonia. It was removed by centrifugation, washed with CO_2-free water, and dried *in vacuo*.

The radioactivities of all samples were measured as described in Paper I (11). The samples were recrystallized or reprecipitated until activities and decomposition points remained constant. None of the tyrosine samples contained detectable radioactivity, and so no further attempt was made to purify these samples.

The glutamic acid hydrochloride and copper aspartate samples were analyzed for their nitrogen content by a modification of the Koch and McMeekin nesslerization method (19). The nitrogen analyses for all samples of glutamic acid hydrochloride ranged from 7.5 to 7.9 per cent (theory for $C_5H_9O_4N \cdot HCl$ 7.6 per cent). The nitrogen analyses for all samples of copper aspartate dried *in vacuo* at room temperature ranged from 4.9 to 5.3 per cent (theory for $C_4H_5O_4NCu \cdot 4\frac{1}{2}H_2O$ 5.09 per cent), and that of two samples dried *in vacuo* at 100° was 7.1 per cent (theory for $C_4H_5O_4NCu$ 7.19 per cent). Since arginine monoflavianate cannot be analyzed well for nitrogen, the criteria of its purity which were used, in addition to constant radioactivity on recrystallization, were decomposition range and the characteristic highly developed luster of the crystals (20).

The radioactivities of the compounds isolated in the first experiment are listed in Table I.

Since the level of C^{14} fixed was low, it was felt that more significant results might be obtained if a higher injection dose were employed, and for this reason a second experiment was carried out. The animals were kept on experimental rations for 15 weeks. Therefore the animal on the low biotin ration had reached a more severe state of biotin deficiency than had been obtained in the previous experiment. The procedure was altered in that a larger total dose of $NaHC^{14}O_3$ was administered as a single injection. The control animal, weighing 356 gm., was injected with a solution of $NaHC^{14}O_3$ equivalent to 10 mg. of barium carbonate having a specific activity of 3 per cent C^{14}. The deficient rat, weighing 198 gm., was given the equivalent of 5.6 mg. of $BaCO_3$. This dose was directly proportional to body weight.

TABLE I

C^{14} Fixed into Tissue Components of Normal and Biotin-Deficient Rats

Tissue	Compound	Isolated and counted as	Activity as net counts per sec. per gm. sample*		
			Ration fed		
			Control	Low biotin	Low biotin-low fat
Liver	Arginine	Flavianate	21	5	2
	Glutamic acid	Hydrochloride	0	0	0
	Aspartic "	Copper salt	5	0	0
Viscera	Arginine	Flavianate	5	6	0
	Glutamic acid	Hydrochloride	0	0	0
	Aspartic "	Copper salt	0	0	0
Muscle	Arginine	Flavianate	3	0	0
	Glutamic acid	Hydrochloride	0	0	0
	Aspartic "	Copper salt	0	0	0
Skeleton	CO_2	Barium "	24	16	
	Citric acid	Calcium "	2	0	

* All activities under 1 count per second per gm. of sample are recorded as zero.

Respiratory carbon dioxide was collected for alternate 15 minute periods, beginning 15 minutes after the injection and continuing until the animal was sacrificed 3 hours later. The animal was placed in a large desiccator into which CO_2-free air was drawn. The expired carbon dioxide was collected in carbonate-free sodium hydroxide solution and precipitated by the addition of a solution of barium chloride. The barium carbonate was centrifuged, washed with CO_2-free water until the washings were neutral to litmus, and dried *in vacuo*. The activities are given in Table II. These experiments were done before it was known that a normal animal injected with less than 1 mg. of $C^{14}O_2$ as $NaHCO_3$ will excrete over 50 per cent of the dose within 16 minutes as respiratory CO_2 (21). It seems probable from our results that the biotin-deficient rat may have lost an even greater percentage of the injected C^{14} during the first 15 minutes after the injection.

When the animals were sacrificed 3 hours after the injection, their tissues were treated in the manner already described for the isolation of tyrosine, arginine, and the barium salts of glutamic and aspartic acids.

TABLE II

C^{14} Concentration of Respiratory CO$_2$ of Animals Given Single Intraperitoneal Injection of NaHC^{14}O$_3$

Sample No.	Min. after injection	Activity as net counts per sec. per gm. BaCO$_3$	
		Control	Low biotin
1	15- 30	47,400	23,500
2	45- 60	28,000	13,400
3	75- 90	19,200	8,300
4	105-120	13,000	6,100
5	135-150	9,400	4,400
6	165-180	6,900	3,400

TABLE III

C^{14} Fixed into Tissue Components of Normal and Biotin-Deficient Rats

Tissue	Compound	Isolated and counted as	Activity as net counts per sec. per gm.*	
			Control	Low biotin
Liver	Arginine	Flavianate	65	11
	Glutamic and aspartic acids	Barium salts	3	0
Viscera	Arginine	Flavianate	7	3
	Glutamic and aspartic acids	Barium salts	3	0
	Adenine	Picrate	29	18
	Guanine	Free base	109	15
Muscle	Arginine	Flavianate	2	2
	Glutamic and aspartic acids	Barium salts	0	0
Skeleton	CO$_2$	Barium salt	260	75
	Citric acid	Calcium "	7	0

* All activities under 1 count per second per gm. of sample are recorded as zero.

When the latter barium salts were being repurified prior to the isolation of the two amino acids, the samples were lost because of an accident while they were being centrifuged. For this reason the fixation of C^{14} into the individual amino acids is not known. Bone carbonate and citrate were isolated as before.

In addition, the purine bases were isolated from the hot trichloroacetic acid extract of the viscera protein. This fraction was chosen in preference to the corresponding fraction from liver, since a preliminary measurement on the crude material indicated a greater C^{14} fixation in the viscera nucleic acid. The extract, made strongly acid with HCl, was freed of trichloroacetic acid by ether extraction and concentrated to dryness *in vacuo*. The residue was hydrolyzed by suspending it in methanol and refluxing for 3 hours in the presence of a continuous stream of dry HCl. The hydrolysate was decolorized with norite, concentrated to dryness *in vacuo*, and dried over $CaCl_2$. It was then taken up in anhydrous methanol saturated with dry HCl. The combined adenine and guanine hydrochlorides were filtered and washed rapidly with methanol saturated with equimolar adenine and guanine hydrochlorides. To the mixture was added double its weight of an equimolar mixture of adenine and guanine hydrochlorides as carriers. The adenine was then separated as the picrate and guanine as the free base (22, 23).

The corrected activities of the compounds isolated in the second experiment are listed in Table III.

RESULTS AND DISCUSSION

The earliest suggestion of a physiological function for biotin was made in 1942 by Pilgrim, Axelrod, and Elvehjem (24) when they demonstrated that liver homogenates from biotin-deficient rats had a greatly decreased rate of pyruvate oxidation compared with normal controls. It has since been demonstrated that the failure of certain microorganisms to grow on a biotin-deficient medium is due to their inability to condense CO_2 with pyruvate to form oxalacetate and aspartate. The data in Tables I and III indicate that the biotin-deficient animal did not fix any appreciable amount of $C^{14}O_2$ into bound aspartic or glutamic acid, while the control fixed a small but definite quantity into aspartic acid. This may indicate that the rat synthesizes aspartate more readily than glutamate. This is in agreement with the finding by Rose *et al.* (25) that glutamate added to a mixture of essential amino acids results in growth stimulation, while aspartate has no effect. The low fixation into these two amino acids of both deficient and normal rats may be due to the fact that on a high protein diet they had an adequate dietary supply of aspartic and glutamic acids and were not obliged to synthesize their own. It has been demonstrated in Paper I (11) that aspartate inhibits the fixation of CO_2 by *Lactobacillus arabinosus*, and the same situation may arise here.

Delluva and Wilson (8) have recently shown that the normal rat incorporates more labeled carbon into arginine isolated from liver protein than into either aspartate or glutamate. They interpret this on the basis of the function of arginine in the urea cycle. Our results similarly indicate a high fixation of C^{14} into liver arginine and, as shown in Table III, the control rat fixed about 6 times as much C^{14} into this amino acid as did the the deficient animal. Since the liver is the main organ of urea formation, this suggests that there may be a slower rate of urea formation in the liver of the biotin-deficient rat.

It should be remembered that the arginine isolated was that of tissue protein and not "free" arginine which might, at the moment, be participating in urea formation. The isotopic amino acids formed during metabolism are also considerably diluted with non-isotopic amino acids of dietary origin when they are incorporated into tissue protein. It should also be pointed out that the major portion of the injected CO_2 is excreted as respiratory CO_2. Gould *et al.* (21) have recently reported that over 90 per cent of an injected dose of $NaHC^{14}O_3$ is excreted within 4 hours in this manner.

There was also a marked difference in C^{14} fixed in the purine bases isolated from the viscera nucleic acid. The adenine isolated from the deficient rat contained about one-half as much C^{14} and the guanine about one-seventh as much C^{14} as did the corresponding compounds from the control animal. This ratio of C^{14} in the adenine samples was the same as that in the respiratory CO_2, while the C^{14} ratio in the guanine samples was very different. The fact that there was a much greater fixation of C^{14} into the guanine of the normal rat than into the adenine seems noteworthy. The values reported were checked with different counter tubes on subsequent recrystallizations. Adenine has been considered a precursor of guanine, since dietary adenine is incorporated into nucleic acid adenine and guanine, as demonstrated by Brown *et al.* (26, 27). However, dietary guanine is not incorporated into nucleic acid adenine or guanine (26, 28). One explanation for the much higher C^{14} fixation into guanine may be that the guanine, when once formed from adenine, can undergo CO_2 exchange without complete disruption of the molecule.

It is probable that the C^{14} in the purines is mainly in position 6, and to a lesser extent in position 4, since it has been shown by Buchanan *et al.* (9) that isotopic $C^{14}O_2$ is fixed into these positions in uric acid, which is presumably derived, at least in part, from the purines. These authors suggest a mechanism for the fixation into position 4 on the basis of a condensation of CO_2 with pyruvate and subsequent formation of isotopic lactate. To date there has been no suggestion for a mechanism of CO_2 fixation into position 6. However, once the purines are synthesized, carbon 6 in adenine is fixed by virtue of the amino group attached to it, but it is possible that carbon 6 in guanine might undergo CO_2 exchange. In this connection, the difference in the ratios of adenine C^{14} to guanine C^{14} should be pointed out. The adenine C^{14} to guanine C^{14} ratio for the control animal is 1:3.8, while for the deficient animal it is only 1:0.8. Some type of metabolic CO_2 exchange, as suggested, may account for the lower C^{14} level in the guanine of the deficient rat compared with the adenine from the same animal. In any case, it is apparent that the deficient animal was less capable of utilizing $C^{14}O_2$ for nucleic acid purines than was the normal animal.

From 15 minutes to 3 hours after the administration of the isotopic carbon, the deficient animal consistently excreted about one-half as much $C^{14}O_2$ as respiratory CO_2 as did the control animal. However, there was only about one-third to one-fourth as much C^{14} in the bone carbonate of the deficient animal compared with the control. Therefore, it seems unlikely that the presence of C^{14} in the bone carbonate is due merely to adsorption or simple exchange of CO_2, but it is probable that an enzymatically catalyzed fixation mechanism is involved as well.

There appeared to be no C^{14} in the bone citrate of the deficient animal, whereas that from the control showed a small but definite quantity of isotopic carbon. This would seem to indicate that bone citrate is in dynamic equilibrium with other tissue citrate. The small fixation is probably due to the fact that the major portion of the isotopic citrate formed would be immediately oxidized via the citric acid cycle.

In a continuation of this work, experiments have been carried out[1] on the influence of biotin deficiency on the ability of rat liver to synthesize citrulline from ornithine. These results (to be published later) demonstrate that livers from biotin-deficient animals synthesize citrulline about one-half as rapidly as do normal rat tissues. Liver tissue from rats pair-fed with the biotin-deficient rats synthesized citrulline as rapidly as did the normal controls. Normal rates of citrulline synthesis were also obtained with tissues from vitamin B_6-deficient rats and their pair-fed controls. It seems probable that biotin is required as a coenzyme in several different enzymatic reactions involving the fixation of CO_2, or that it is essential for the formation of a single common precursor which is incorporated into the carbon structure of arginine, aspartic acid, citric acid, and the purines.

We are indebted to Professor R. H. Burris for generously supplying C^{14} and facilities for its determination.

SUMMARY

Intraperitoneal injection of sodium bicarbonate containing C^{14} into normal and biotin-deficient rats resulted in a larger C^{14} fixation into the adenine, guanine, arginine, aspartic acid, citric acid, and bone carbonate

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[1] In collaboration with Dr. S. Grisolia and Professor P. P. Cohen.

of the control animal than into the corresponding compounds of the biotin-deficient animal. The implications of these results are discussed.

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PHOTOMETRIC MEASUREMENT OF PLASMA pH

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The advantages of the spectrophotometric technique in applications of indicator methods were pointed out in 1924 by Brode (1) and by Holmes (2, 3) who emphasized the accuracy of readings, the freedom from error from phenomena of dichromatism, the ease with which corrections can be made for color or turbidity in the material examined, and the small amounts of fluid that can be used. Over comparator technique the photometric has also the advantage that the latter does not require the continuous use of numerous standards.

Nevertheless the spectrophotometer has not found general application in measurement of plasma pH. Perhaps one reason is that the careful studies of Robinson and Hogden (4) on the optical density curves of phenol red in serum, buffered to known pH levels by mixing with phosphate and veronal buffers, showed peculiar effects of the plasma proteins on the density curves of the dye. The wave-length at which the dye showed maximal optical density was shifted from its normal value of 560 mμ, and dye densities measured at 560 mμ were significantly decreased by the presence of the proteins. The effect was greater when phosphate was used than when veronal buffers were employed.

Results in the present paper show that when human plasma is diluted with 20 volumes of neutral 0.9 per cent NaCl solution containing phenol red, the only buffers present being those of the plasma, effects of the proteins on the optical density of the dye, such as those observed by Robinson and Hogden in serum diluted with phosphate or veronal buffer solution, either do not occur, or occur only to such an extent as to balance a slight increase in pH caused by diluting the plasma with the NaCl solution. Consequently it is possible to estimate human plasma pH from the optical density of phenol red in the diluted plasma with a standard deviation, from the pH determined in undiluted plasma by the hydrogen electrode, of only ±0.02 pH unit.

The indicators, temperature control, and conditions for diluting the plasma are the same as those used by Hastings and Sendroy (5) in colorimetric plasma pH measurements by means of test-tube comparators, but changes in so many details have been found expedient in adapting the procedure to the spectrophotometer that their description appears desirable.

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Relation of Optical Density to pH and pK′ of Indicator

With the sulfonphthalein indicators (4–8) it has been found that the relation of optical density to pH can be formulated, for the useful ranges of the indicators, on the assumptions (1) that the indicator is divided into the undissociated acid, HA, and the anion, A^-, according to the Henderson-Hasselbalch equation for weak acids, the anion A^- representing the dissociated alkali salt of the indicator, and (2) that each of the two forms of the indicator has its own characteristic curve of optical density *versus* wave-length of transmitted light, the densities of the two forms being additive.

Let E_1 and E_2 be the optical densities of the alkaline and acid forms of the indicator, respectively, measured in solutions of unit concentration (*e.g.*, 1 mg. or 1 mM per liter), and with a transmitting layer 1 cm. in length. Let D be the measured density at any given pH in the useful range of the indicator, in a solution of S concentration of total dye, with a transmitting layer of l cm. Let f be the fraction of the indicator in the alkaline form, A^-, and $1-f$ be the fraction in the acid form, HA. Then, *for light of a given wave-length,*

$$D = lE_1fS + lE_2(1-f)S \tag{1}$$

When D_1 and D_2 are the optical densities of the alkaline and acid forms, respectively, of the indicator measured in a cuvette presenting the same l used to measure D, and in a solution of the same concentration, S, used to measure D, the equation simplifies to

$$D = D_1f + D_2(1-f) \tag{2}$$

The Henderson-Hasselbalch equation, $\text{pH} = \text{pK}' + \log([A^-]/[HA])$ may be written as $\text{pH} = \text{pK}' + \log(f/(1-f))$. From Equation 2, $f = (D - D_2)/(D_1 - D_2)$. Whence $(f/(1-f)) = (D - D_2)/(D_1 - D)$, and

$$\text{pH} = \text{pK}' + \log\frac{D - D_2}{D_1 - D} \tag{3}$$

When, at the wave-length used, $D_2 = 0$ (*e.g.*, phenol red with wave-length 550 to 560 mμ (4)), Equation 3 simplifies to

$$\text{pH} = \text{pK}' + \log\frac{D}{D_1 - D} \tag{4}$$

The above formulation applies to the method described in the present paper.

In the application of Equations 3 and 4 to pH measurement, requirements for accuracy are that the measurements of D be made with solutions of the same dye concentration, S, and in cuvettes presenting the same l, used in determining the constants D_1 and D_2; accurately calibrated cuvettes and accurately measured additions of dye are therefore essential.

With some indicators the necessity for constant l and S can be avoided by measuring in the same cuvette two optical densities, D_a and D_b, at two different wave-lengths, a and b, such that the pH is indicated by the ratio $D_a:D_b$. The theoretical basis of the procedure may be formulated from Equation 1 as follows, subscripts a and b being used to indicate values at the wave-lengths a and b respectively.

$$\frac{D_a}{D_b} = \frac{Sl[fE_{a1} + (1 - f)E_{a2}]}{Sl[fE_{b1} + (1 - f)E_{b2}]} \tag{5}$$

The values of S and l cancel out, and the ratio $D_a:D_b$ becomes the measure of f and hence of pH. The curve of $D_a:D_b$ *versus* pH can be plotted by calculating f for values of pH calculated by the Henderson-Hasselbalch equation in the form, $f = 1/(1 + \text{antilog}\ (\text{pK}' - \text{pH}))$.

Freedom from necessity for accurate addition of dye to obtain a constant S value may offer a significant advantage when small volumes are used. On the other hand, the ratio procedure has the disadvantage that it requires two readings, so that their errors are additive. The procedure has recently been used by Rutledge (7) for plasma pH determinations with phenol red, with measurement of densities at the wave-lengths 565 and 420 mμ, at which maximal densities are approached by the alkaline and acid forms of the indicator, respectively.

Special Apparatus

A photometer, preferably capable of receiving cuvettes in the form of test-tubes.[1]

A small water bath set at 38.5° and provided with a rack to hold six or more cuvettes.

Cuvettes, cylindrical, of about 16 mm. bore for macrodetermination, and about 10 mm. for microdetermination.[2]

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[1] The present work was done chiefly with the Coleman junior spectrophotometer, Coleman Electric Company, Maywood, Illinois.

[2] As cuvettes we have used "culture" test-tubes, No. 9446 of the Arthur Thomas Company, 150 × 18 mm. external measurement (16 mm. bore) for macrodeterminations, 75 × 12 mm. (10 mm. bore) for microdetermination. The cuvettes of each set were tested for uniformity by measuring in them the optical density of a solution of phenol red, 2 mg. per liter, in 0.002 N NaOH, with light of 550 mμ. The solution showed an optical density of about 0.40 in the larger cuvettes and 0.23 in the micro cuvettes. A set of each size is selected such that each tube of a set gives an optical density within ±0.5 per cent of the mean and shows no change when the tube is rotated. From 100 tubes it is usually possible to select a set of twenty which meet these requirements. We have found that this selection yields more uniform cuvettes and is more economical than the purchase of calibrated cuvettes.

A 2 ml. graduated pipette drawn out near the tip into a capillary about 30 mm. in length and 1 to 1.5 mm. in bore. The capillary is used for delivering plasma into saline-dye solution under a layer of oil.

Permanent Solutions

Stock Solutions of 0.5 M Na_2HPO_4 (142.0 Gm. per Liter) and KH_2PO_4 (136.1 Gm. per Liter)—Anhydrous salts are used. The solutions are kept in Pyrex flasks in an ice box. The stock solutions are made of 0.5 M instead of M/15 concentration because the latter are less stable. From the Na_2HPO_4 solution crystals separate in the cold, but they are readily redissolved by warming each time the solution is used. The solutions in stoppered Pyrex flasks keep unchanged in the refrigerator for at least 1 year. The Na_2HPO_4 solution will absorb atmospheric CO_2 and change its pH value if much exposed to air. Hence the stoppers are removed from the flasks only for the intervals necessary to withdraw solution for use.

TABLE I

Preparation of Standard 0.5 M Phosphate Solutions

0.5 M stock solution		pH obtained at temperature indicated when mixed 0.5 M phosphate is diluted to M/15			
Na_2NPO_4	KH_2PO_4	20°	26°	32°	38°
ml.	*ml.*				
50.0	23.68	7.13	7.12	7.11	7.10
50.0	11.48	7.43	7.42	7.41	7.40
50.0	5.57	7.73	7.72	7.71	7.70

0.5 M Mixed Phosphate Solutions—These three solutions are made by mixing the above stock solutions in the proportions shown in Table I. Into each of three Pyrex Erlenmeyer flasks of 125 ml. capacity pipette 50 ml. of the 0.5 M Na_2HPO_4 solution. Then from a 25 ml. burette measure the volumes of 0.5 M KH_2PO_4 solution indicated in Table I. The solutions will keep unchanged for a year if kept stoppered in a refrigerator. They are used for preparing the less stable M/15 standard phosphate solutions.

The proportions of Na_2HPO_4 and KH_2PO_4 for different pH values are interpolated from the data of Hastings and Sendroy (5) who determined with the hydrogen electrode at 20° and 38° the pH values of twenty-five mixtures of M/15 phosphates over the pH range from 6.8 to 8.1, their electrodes being standardized with 0.1 N HCl, which was assumed to have a pH of 1.08 at both temperatures.[3] Because of the small and linear change of the pK′ of phosphate with pH, the simplest procedure to obtain exact interpolation, and to detect deviations of individual data from the trend of the series, is to plot the best line of the equation, $pK' = a + b\,pH$, and estimate values of pK′ for desired pH values by interpolation on this line. The best lines for 20° and 38° are indicated by the equations

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[3] The reasons for the utility of 0.1 N HCl, with assumed pH of 1.08, as a standard have been presented by Cullen, Keeler, and Robinson (9), on grounds that appear to be still valid. This standard is the present basis of many of the data used in biochemistry, such as the values of the pK′ of the Henderson-Hasselbalch equation used

$$pK'_{20^\circ} = 7.125 - 0.045\ pH_{20^\circ} \tag{6}$$

$$pK'_{38^\circ} = 7.107 - 0.0467\ pH_{38^\circ} \tag{7}$$

Of the 50 sets of Hastings and Sendroy's values, all but two at 20° and one at 38° gave values for pK′ within ±0.004 unit of those indicated by Equations 5 and 6.

From these equations R, the ratio $Na_2HPO_4:KH_2PO_4$, of the Henderson-Hasselbalch equation, $pH = pK' + \log R$, is defined for M/15 phosphate solutions at the two temperatures by Equations 8 and 9.

$$\log R_{20^\circ} = 1.0450\ pH_{20^\circ} - 7.125 \tag{8}$$

$$\log R_{38^\circ} = 1.0467\ pH_{38^\circ} - 7.107 \tag{9}$$

When 50 ml. of Na_2HPO_4 solution are used, the ml. of KH_2PO_4 are calculated for any pH as $50/R$.

Phenol Red, Stock 0.1 Per Cent Solution—Grind 100 mg. of phenol red with 28.2 ml. of 0.01 N NaOH till dissolved, and dilute to 100 ml. (6). For several days after the solution is prepared the phenol red may undergo change, indicated by a shifting of the pH-optical density curve prepared with solutions of the dye in phosphate buffers. Hence, in order to have a stock solution from which working solutions of relatively constant optical properties can be prepared, it is preferable to prepare the stock solution several days before use of it is begun.

Phenol Red Solution, 80 Mg. per Liter—Dilute 20 ml. of 0.1 per cent phenol red solution to 250 ml. with water. Store in a Pyrex flask in the refrigerator. It will usually keep several months without change in optical density values. The stability of the dye is checked by repeating the determination of the standard curve of pH *versus* optical density (see below) once a month or oftener.

NaCl Solution, 22.5 Per Cent (225 Gm. per Liter).

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for bicarbonate solutions (10), for blood plasma (9, 11, 12), and for urine (13). pH values obtained on the basis of this standard agree nearly within the limit of experimental error with values at 18° based on the original phosphate buffer solution of Sørensen (14). 0.1 N HCl was used as standard by Sendroy, Shedlovsky, and Belcher (15) together with the acetate standards of MacInnes, Belcher, and Shedlovsky (16). Liquid junction potential made the 0.1 N HCl give E_0 values less accurately reproducible than values by the acetate standards in the glass electrode. In the Clark type of hydrogen electrode, however, the HCl values were found reproducible, and, in agreement with the acetate values, to less than 0.01 pH (MacInnes, personal communication).

NaCl Solution, 0.9 Per Cent—Dilute 10 ml. of the 22.5 per cent solution to 250 ml.

Neutralized Mineral Oil—In a 500 ml. separatory funnel place about 200 ml. of mineral oil, an equal volume of water, and a few drops of 0.1 per cent phenol red solution. Add 0.02 N NaOH a drop at a time with vigorous shaking until the water solution of indicator becomes permanently pink. Centrifuge the oil to get out suspended water droplets. Decant the clear oil with care that none of the water from the bottoms of the centrifuge tubes is mixed with the oil. Store in a stoppered flask.

Approximately 0.02 N NaOH, kept in a Pyrex vessel and protected from exposure to atmospheric CO_2.

Solutions Made Immediately before Use

Neutralized Saline-Dye Solution with 8 Mg. of Phenol Red per Liter—This solution is prepared immediately before it is to be used, as the pH is likely to fall too much if the solution stands for more than an hour or two. In a 100 ml. volumetric flask place 4 ml. of the 22.5 per cent NaCl solution and about 80 ml. of water, and measure in accurately 10.00 ml. of the 80 mg. per liter phenol red solution. Add by drops 0.02 N NaOH solution, stirring by rotating the flask after each addition, until the color of the solution, judged by the eye, indicates that the pH is approaching 7.4. Then fill the flask to within about 0.2 ml. of the mark, and continue addition of the 0.02 N NaOH a drop at a time until the pH at room temperature is between 7.4 and 7.6. The pH is tested at this stage after each addition of NaOH by pouring about 10 ml. of the solution into a cuvette and reading quickly the optical density. The solution is then returned to the 100 ml. flask, and, if necessary, more 0.02 N NaOH is added. This procedure is repeated until the pH is raised to between 7.4 and 7.6. It is better to have it a little above 7.4 rather than below, because the pH tends to fall from absorption of atmospheric CO_2 while the solution is being used. If the pH falls below 7.4 before a series is finished, it may be restored by adding a drop of 0.02 N NaOH to the unused residue of the solution. Keep the 100 ml. flask containing the solution stoppered to retard absorption of atmospheric CO_2. As shown by Hastings and Sendroy (5), the accuracy of the colorimetric pH determination is not significantly affected if the pH of the saline dye solution is within ±0.2 pH unit of the plasma pH.

M/15 Standard Phosphate-Dye Solutions of pH 7.10, 7.40, and 7.70 (at 38°) Containing 7.62 Mg. of Phenol Red per Liter—(These are needed only when the pH-optical density curve is checked.) For standards for the macroprocedure described below, prepare six dry test-tube cuvettes with stoppers. Duplicate cuvettes are prepared with each of the three phosphate solutions of Table I. Into each cuvette measure 8.15 ml. of water, 1.35 ml. of 0.5 M mixed phosphate solution (Table I), and, from a calibrated pipette, 1.000 ml. of the 80 mg. per liter phenol red solution, making a total volume of 10.5 ml. After adding the dye solution, mix the contents of each cuvette with a footed rod and at once stopper the cuvette to protect from atmospheric CO_2.

If the microprocedure is to be used, one solution of each pH is prepared as above, and duplicate portions of each are pipetted into micro cuvettes.

The solutions with the three pH values are used on the same day on which they are made.

Preparation of Standard pH-Optical Density Curves

Prepare three pairs of cuvettes with M/15 phosphate of $pH_{38°}$ 7.10, 7.40, and 7.70, respectively, as described above.

Two curves are prepared, one with the standard phosphate-dye solutions at room temperature, the other with the same solutions warmed to 38°. The six tubes provide duplicate readings for each pH point.

For the room temperature curve the optical densities of the freshly prepared standard phosphate-dye solutions are read in the photometer, the zero point being set with a water blank before each reading. (This curve is used only for checking the approximate pH of saline-dye solutions.)

For the 38° curve the six cuvettes containing the standard phosphate-dye solutions are placed in a bath of water of temperature 38.5°. The cuvettes are immersed so that the surfaces of their solutions are below the level of the water surface in the bath. 5 minutes in the bath suffice to bring the solutions to its temperature. Each tube is removed from the bath, wiped, and its optical density is quickly read with a wave-length of 550 mμ before its temperature falls below 38°. (1° temperature change alters the apparent pH by about 0.01 unit.) The zero point of the photometer is set before each reading with a water blank, which need not be warmed.

The densities for the 38° curve are plotted for the pH values 7.10, 7.40, and 7.70; the densities for room temperature are plotted for the pH values indicated by Table I. Since the curves are nearly linear, three points suffice for each. They are plotted on a scale which permits estimating densities to ±0.001 and pH to ±0.002. Fig. 1 shows an example of the curve obtained with cuvettes of approximately 16 mm. bore.[2]

MACROPROCEDURE FOR DETERMINATION OF PLASMA PH

Preparation of Duplicate Saline Plasma-Dye Solutions and of Blank Solution—Draw about 5 ml. of blood without exposure to air and run the blood under oil previously placed in a tube that contains a dry film of heparin

(0.2 mg. per ml. of blood) or of neutral potassium oxalate (1 to 2 mg. per ml. of blood).

Centrifuge under conditions avoiding loss of CO_2. Blood centrifuged under a thick layer of oil may lose enough CO_2 to the oil to raise appreciably the pH of the plasma. A convenient device for anaerobic centrifugation is the float shown in Fig. 2.[4] The float is inserted after the blood has been

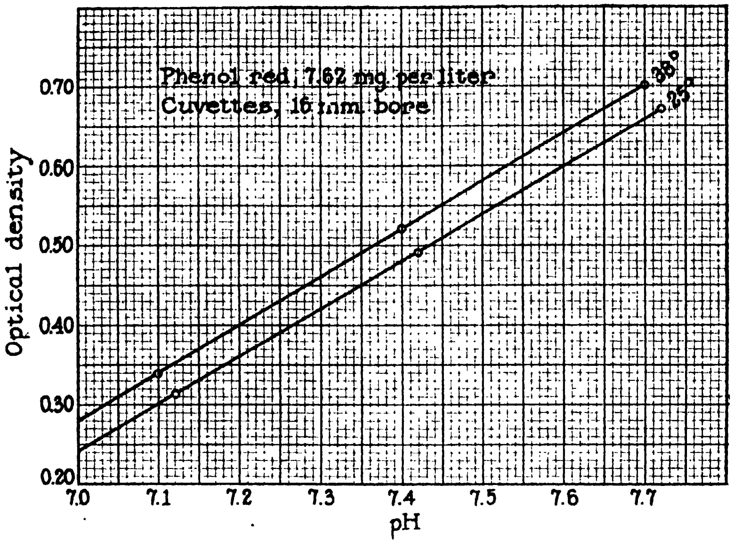

Fig. 1. Type of nearly linear curve of optical density of phenol red, measured with light of 550 mμ wave-length, at pH 7.1 to 7.7.

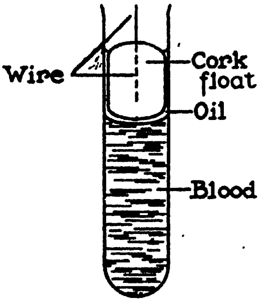

Fig. 2. Test-tube cuvette with cork float, oil, and blood, prepared for anaerobic centrifugation. See foot-note 4.

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[4] The float is of a diameter about 1 mm. less than the bore of the centrifuge tube. The float may be made of thin glass tubing, a flattened nob being left at the top to be grasped by pincers when the float is withdrawn from the tube. The float may also be made of cork, by grinding a stopper to a cylinder of the necessary diameter, rounded at both ends as shown in Fig. 2. To facilitate withdrawing the cork from tubes, a thin copper wire is forced part way into the cork, as shown in Fig. 2.

run under the oil and mixed with the anticoagulant. Then, by gently pressing a capillary tube attached to suction against the upper edge of the float, oil is withdrawn until the bottom of the float sinks nearly to the surface of the blood, leaving a layer of oil only 1 or 2 mm. thick between float and blood. The tube is centrifuged with the float in place. Just before plasma is withdrawn for the pH determination, the float is removed from the tube, leaving a thin layer of oil, which protects the blood during the time required for pipetting out the plasma sample.[5]

Pipette two 10.00 ml. portions of the neutralized saline-dye solution into two cuvettes, for duplicate determinations, and immediately cover with neutralized mineral oil. In a third cuvette place 10 ml. of 0.9 per cent NaCl solution without dye or oil to receive plasma for the plasma blank.

Draw 1.6 ml. or more of plasma into the 2 ml. graduated pipette, the tip of which is drawn out into a capillary (see "Special apparatus").

Run 0.5 ml. of plasma into each of the two cuvettes containing 10.00 ml. of saline-dye solution under oil, the drawn out tip of the pipette being thrust through the oil into the saline-dye solution during the delivery.

A third 0.5 ml. portion of plasma, to serve as a blank, is then delivered into the cuvette that contains 10 ml. of 0.9 per cent NaCl without dye or oil.

Mix the solutions in all three cuvettes by stirring with clean, footed rods.

Place all the cuvettes in a water bath at 38.5°. Begin readings after the cuvettes have been in the bath 5 minutes.

The use of duplicate solutions of plasma plus dye adds security against error from contaminated or inaccurately calibrated cuvettes. The readings of the duplicates seldom differ by more than 0.01 pH, however, and the duplicate plasma solution may be omitted when plasma for it is lacking.

Photometric Readings—The optical density, D, of the dye in the dye-plasma solution is read, with the dye-free plasma-saline solution as a blank. Readings are made with the solutions at 38°, as described for readings of the phosphate-dye solutions in preparing the standard curve.

In a photometer in which the blank and unknown are read in succession with the cuvettes in the same light beam, it is convenient, for a series of readings, to set the zero point with a water blank, and read the densities of both the plasma-saline blank and the plasma-dye solution, rather than to reset the zero of the photometer with each plasma blank. Then the dye density, D, is calculated as $D = D_1 - D_2$, where D_1 is the density of the plasma-dye solution and D_2 is the density of the plasma blank. The setting of the zero with the water blank is checked with each plasma reading.

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[5] In a recent paper Rosenthal (17) has found that in order to obtain pH values for plasma exactly equal to those of the blood as drawn it is necessary to centrifuge at 38°. If the blood was cooled to 20° and centrifuged at that temperature, and the separated plasma was then warmed back to 38° for pH measurement, the pH obtained was about 0.03 unit greater than that observed if the centrifugation was carried out at 38°. However, practically all the data in the literature on the pH of blood plasma under physiological and pathological conditions have been obtained from plasma centrifuged at room temperature, and, for purposes of comparison with these data, it appears justifiable to continue the practice of centrifuging at room temperature.

Calculation

The pH is estimated by interpolation of D on the standard curve of pH *versus* D at 38°.

Blank for Optical Density of Plasma—The D_2 values obtained with normal, apparently clear plasmas in the cuvettes of 16 mm. bore have varied between 0.01 and 0.04 optical density units, sufficient to affect the estimated pH by 0.015 to 0.06. In such plasmas, from normal blood not lipemic from recent meals, one could, in case of necessity from lack of material, omit the D_2 reading and subtract 0.04 pH unit from the pH estimated from the D_1 reading, with a resultant error not exceeding ±0.02 pH unit. After high fat meals have produced dense lipemia, however, D_2 values as high as 0.11 have been observed in 21-fold diluted normal plasma, and some pathological lipemias gave even higher values. These did not appear to affect the accuracy of the pH determinations when the plasma blanks were handled as described for the method.

MICROPROCEDURE FOR PLASMA PH

The procedure is identical with that described above, except that cuvettes of approximately 10 mm. bore[2] are used, and one-fifth the volumes of plasma and saline-dye solution taken for the macroprocedure, the amount for each cuvette in the micromethod being 0.1 ml. of plasma and 2 ml. of the saline-dye solution.

MICROPROCEDURE WITH WHOLE BLOOD

As shown by Hawkins (18), and confirmed by Hastings and Sendroy (5), if whole blood is mixed with 10 times its volume of neutral saline-dye solution and immediately centrifuged, estimation of the pH from the color of the supernatant solution gives the same results as those obtained by first separating the plasma and then diluting it with 20 volumes of the saline-dye solution, as in the procedures described above. We have performed a few comparative analyses which indicate that similar identity of results is obtained when the measurements are based on optical densities.

The reagents are the same as for plasma, except that the dye in the saline-dye solution mixed with the blood is made 5 per cent stronger (5). Hence, instead of diluting 10 ml. of the 80 mg. per liter dye solution to 100 ml., 10.5 ml. are diluted to 100 ml. to prepare the saline-dye solution.

Procedure

0.2 ml. of whole blood is run into a micro cuvette containing 2 ml. of neutral saline-dye solution, which is covered with a few mm. of oil and mixed with the blood. The oil is replaced by low melting paraffin, and the tube is at once centrifuged. The blood blank is prepared in the same way, except that the blood is mixed with 0.9 per cent NaCl containing no dye.

The centrifuged cells should settle so closely to the bottom of the cuvette that they are below the path of light in the photometer. If this is not the case, the bottom of the slit in the adapter, in which the cuvette rests during readings, is covered with black paper to a sufficient height to insure that the cells will remain below the path of light. The photometer readings must be made within a half hour after centrifugation, or plasma pH may fall from acid formed in the cells.

EXPERIMENTAL

Determination of Plasma pH with Hydrogen Electrode

Preparation of H_2-CO_2 Mixtures—A low pressure steel tank was connected through heavy walled rubber tubing to a T-tube connected through stop-cocks to a flask of solid CO_2 and to a high vacuum pump. To remove air the tank was alternately evacuated and allowed to fill to atmospheric pressure with CO_2 three times. It was then evacuated again, and sufficient CO_2, measured by pressure, was admitted to give the desired amount in the final CO_2-H_2 mixture. The tank was then coupled to a high pressure tank of hydrogen and loaded to a total of 50 pounds positive pressure. Samples of the gas were withdrawn over mercury and analyzed for carbon dioxide content by the isolation method of Van Slyke, Sendroy, and Liu (19). Mixtures were thus prepared of the composition indicated in Table II.

Preliminary Equilibration of Plasma with H_2-CO_2 Mixture—10 ml. of fresh plasma were introduced into a 500 ml. separatory funnel fitted at the mouth with a tube closed with a pinch-clamp on rubber tubing. The funnel was evacuated with a water pump and filled with hydrogen-carbon dioxide mixture from the tank. The funnel was transferred to the 38° room and allowed to attain atmospheric pressure by quickly opening and closing the stop-cock as the gas warmed. Equilibration with the gas was obtained by rotating the funnel and passing fresh H_2-CO_2 mixture through it.

pH Measurement in Clark Cells—Four Clark (20) cells (Cullen modification (21)) were connected through saturated KCl liquid junction to a saturated KCl-calomel electrode and each was filled completely with the previously equilibrated plasma. To assure complete equilibration fresh gas mixture was passed through each cell for 1 minute, displacing part of the plasma. The cell was then closed, except for the hydrogen inlet tube, and was rocked for 15 minutes with the hydrogen-carbon dioxide mixture at atmospheric pressure.

TABLE II

Comparison of Electrometric and Photometric pH Values at 38°

Plasma No.	Photometric, pH_P	Electrometric		$pH_H - pH_P$	CO_2 content of H_2 used to saturate plasma	Acid or alkali added to plasma
		Hydrogen electrode, pH_H	Glass electrode, pH_G			
					per cent	
1	7.60	7.58	7.60	−0.02	4.90	0
2	7.32	7.32	7.34	±0.00	4.90	0
3	7.21	7.20	7.20	−0.01	6.22	0
4	7.23	7.22	7.19	−0.01	6.22	0
5	7.24	7.24	7.23	±0.00	6.22	0
6	7.14	7.13		−0.01	6.22	0.025 vol. 0.2 N lactic
7	7.63	7.63	7.65	±0.00	1.97	0
8	7.63	7.65	7.65	+0.02	1.97	0
9	7.35	7.37		+0.02	1.97	0.025 vol. 0.2 N lactic
10	7.36	7.33		−0.03	5.25	0
11	7.43	7.47	7.47	+0.04	4.18	0
12	7.50	7.54	7.52	+0.04	4.18	0
13	7.51	7.52		+0.01	4.18	0
14	7.53	7.53		±0.00	4.18	0
15	7.44	7.46	7.43	+0.02	4.18	0
16	7.17	7.19		+0.02	4.18	0.015 vol. 1 N lactic
17	7.34	7.36		+0.02	6.65	0
18	7.65	7.64	7.63	−0.01	6.65	0.04 vol. 1 N NaOH
19	7.27	7.27	7.27	±0.00	4.48	0.015 " 1 " HCl
20	7.07	7.03	7.01	−0.04	4.48	0.025 " 1 " "
21	7.83	7.82		−0.01	4.48	0.04 " 1 " NaOH
22	6.93	6.89		−0.04	5.82	0.025 " 1 " HCl
23	7.68	7.68		±0.00	5.82	0.04 " 1 " NaOH
24	7.39	7.40	7.42	+0.01	5.82	0
25	7.16	7.18	7.17	+0.02	5.82	0.015 vol. 1 N HCl
26	7.46	7.47		+0.01	5.82	0
Average deviation				+0.002		
Standard "				±0.022		
Maximal "				±0.04		

Potentials were measured to the nearest 0.1 mv. by a type K Leeds and Northrup potentiometer and a sensitive Leeds and Northrup galvanometer. Usually all four cells gave similar results. If one deviated markedly from the other three, it was discarded in calculating the average.

All measurements were carried out in a room at a constant temperature of 38°. The actual temperature at the electrode was measured and the necessary corrections were applied to the pH calculation.

The platinum black electrodes were prepared with a heavy coating of platinum over gold over smooth platinum. It was generally necessary to replate after two determinations of plasma pH.

The hydrogen electrodes were standardized at 38° with each CO_2-H_2 gas mixture against 0.1000 N hydrochloric acid, the pH of which was assumed to be 1.08.[3] All measurements were corrected for actual hydrogen pressure, as calculated from the barometric pressure, the H_2 content of the H_2-CO_2 mixture, and the vapor pressure of water at 38°.

Determination of Plasma pH with Glass Electrode

After the completion of the hydrogen electrode measurements a sample of the plasma was withdrawn from a Clark cell directly into a MacInnes-Belcher (22) electrode, modified by Michaelis (23), which was fitted at the base with a stop-cock. Liquid junction to saturated KCl was established by immersing the end of the electrode in a beaker of KCl connected with a calomel half cell. The pH was determined with a vacuum tube electrometer of a type similar to that of the Cambridge Instrument Company. The entire operation was carried out in the 38° constant temperature room. The glass electrode was standardized against two known M/15 phosphate solutions, one of pH above, the other below, that of the plasma.

Determination of Plasma pH with Photometer

After the measurements with the hydrogen electrodes were completed, a 2.0 ml. graduated pipette, fitted with a bent capillary at the tip for entering the Clark cells, was filled with plasma from one of the cells. Three portions of 0.5 ml. each were run into cuvettes containing 10 ml. portions of saline-dye solution, as described for the macromethod. The fourth portion of 0.5 ml. of plasma was used for the plasma blank. The pH determinations, in triplicate, were made as described for the photometric procedure. The three values of each set usually agreed within 0.01 pH unit.

RESULTS

Comparative results by the three procedures are given in Table II. Some of the plasmas were mixed with acid or alkali, as indicated in Table II, to simulate conditions with low or high alkali reserve. In Plasmas 7, 8, and 9 the CO_2 tension was lowered, to give the conditions of a respiratory alkalosis in Plasmas 7 and 8, and of a compensated low alkali reserve in Plasma 9.

Both the observed maximal deviation between pH values obtained by the hydrogen electrode and the photometer, respectively, and the maximum estimated as twice the standard deviation indicate a deviation of ±0.04 as the maximum. Of the twenty-six plasmas, twenty-one showed deviations not exceeding 0.02 pH unit.

SUMMARY

The effects of plasma proteins on the optical density of phenol red, observed by Robinson and Hogden in plasma diluted with phosphate or veronal buffers, are found to be negligible in plasma diluted with 20 volumes of 0.9 per cent NaCl solution.

Procedures are described for spectrophotometric measurement of plasma pH in samples of 1.0 to 0.2 ml. of blood or plasma. The standard deviation from pH values measured by the Clark hydrogen electrode was ±0.02 pH.

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PHOTOMETRIC MEASUREMENT OF URINE pH

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The principles of photometric pH determination have been discussed in the preceding paper (1). The conditions used for urine are essentially those devised for the visual colorimetric method of Hastings, Sendroy, and Robson (2), except that (1) the Duboscq type colorimeter is replaced by a spectrophotometer, (2) lower dye concentrations are used in the cases of brom-cresol purple and brom-cresol green, and (3) for buffer standards over the pH range from 4.9 to 5.8 acetate solutions of 0.05 M concentration are used, prepared from the pK′ values of MacInnes, Belcher, and Shedlovsky (3), instead of the 0.2 M acetate standards of Walpole (4). In comparing the optical densities of solutions of brom-cresol purple in phosphate and acetate buffers of pH 5.8, at which the useful pH ranges of acetate and phosphate buffers overlap, it was found that when 0.2 M acetate was used the density of the dye in the acetate exceeded the density in M/15 phosphate by an amount corresponding to a difference of 0.04 pH. The difference is presumably attributable to the effect on the dye of the greater ionic strength of the 0.2 M acetate. When acetate of 0.05 M concentration was used, the dye in it showed the same optical density as in M/15 phosphate of the same pH.

Permanent Solutions

Stock 0.5 M Na_2HPO_4 and KHP_2O_4—These are prepared and kept as described for plasma pH determinations (1).

Stock 0.5 M Sodium Acetate and Acetic Acid Solutions—The concentration of the acetic acid is checked by titration against standard NaOH solution. The sodium acetate solution may be prepared from $NaC_2H_3O_2 \cdot 3H_2O$ (68.03 gm. to a liter) of tested Na content, or it may be prepared by mixing equal volumes of 1 N NaOH and 1 N acetic acid that have been shown by titration against each other to be of equal normality.

Indicator Solutions—Solutions containing 1 mg. of dye per ml. are prepared by adding to 100 mg. of each dye the volume of 0.01 N NaOH indicated by Table I and diluting to 100 ml. From these stock solutions portions of 6 and 8 ml. respectively are diluted to 100 ml. to make solutions containing 60 and 80 mg. of dye per liter.

All the permanent solutions are kept in a refrigerator when not in use.

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Solutions Prepared Fresh before Using

M/15 Phosphate Buffer Solutions—These solutions are prepared from the 0.5 M solutions as indicated by Table II. The proportions of Na_2HPO_4 and KH_2PO_4 are calculated from the pK′ values of Equation 7 of the preceding paper (1).

0.05 M Acetate Buffer Solutions—These are prepared as indicated by Table III. The acetate values given in Table III are calculated from the pK′ data of MacInnes, Belcher, and Shedlovsky (3). The data of these authors show that the relation between ionic strength, μ, and pK′ of acetate buffer solutions can be expressed by the equations, $pK' = 4.769 - 0.5774\sqrt{\mu}$ at 38° and $pK' = 4.756 - 0.5774\sqrt{\mu}$ at 25°.

TABLE I

Indicators for Urine pH

Indicator	pK′ of indicator at 38° (Hastings *et al.* (2))	pH range used for urine	0.01 N NaOH added to 100 mg. indicator to make Na salt (Clark (5))	Concentration of indicator used		Wave-length of light used	Approximate optical density of dye in cuvette of 16 mm. bore		Approximate mean change in density caused by change of 0.01 in pH
				In solution added to 10 volumes diluted urine	In final urine-dye solution		At lowest pH of range	At highest pH of range	
			ml.	*mg. per l.*	*mg. per l.*	*mμ*	*D*	*D*	*D*
Brom cresol green	4.72	4.9–5.8	14.3	80	7.27	620	0.33	0.57	0.0027
" " purple	6.09	5.8–6.9	18.5	60	5.45	590	0.28	0.71	0.0039
Phenol red	7.78	6.9–8.1	28.2	80	7.27	550	0.20	0.76	0.0046

Preparation of Standard pH-Optical Density Curves

Standard solutions are made by adding portions of 1 ml. of dye solution (60 or 80 mg. per liter) to 10 ml. portions of 0.05 M acetate or M/15 phosphate buffer solutions. The range covered by each dye is indicated by Table I. The buffer-dye solutions in test-tube cuvettes are warmed to 38° and the optical densities read as described for preparation of the optical density curves for plasma determinations (1).

The stabilities of the indicator solutions are checked once a month or oftener by repeating the density measurements with each indicator at one pH; *e.g.*, pH 5.5 for brom cresol green, pH 6.2 for brom cresol purple, pH 7.2 for phenol red. If the optical density shows a significant change, the entire curve for the indicator is redetermined.

PROCEDURE FOR DETERMINATION OF URINE PH

The urine is drawn and kept with precautions to prevent loss of CO_2, and the pH is determined as soon as possible, preferably within 2 hours.

Into uniform cuvettes, of the type described for macrodeterminations of plasma pH (1), are measured 8 ml. portions of distilled water, 2 ml. portions of urine, and 1 ml. of dye. The fluids are mixed with a footed rod, covered with neutral oil, and warmed to 38°, as described for plasma determinations (1). For each urine a blank is prepared by diluting 2 ml. of urine with 9 ml. of water. The optical densities are read as described for plasma pH (1).

TABLE II

*M/15 Phosphate Buffers**

pH at 38°	Volumes to make 10 ml. buffer solution		Volumes when larger fraction is 25 ml.	
	Na_2HPO_4	KH_2PO_4	Na_2HPO_4	KH_2PO_4
	ml.	*ml.*	*ml.*	*ml.*
5.8	9.16	0.84	25.0	2.29
6.0	8.70	1.29	25.0	3.71
6.3	7.65	2.35	25.0	7.68
6.6	6.13	3.87	25.0	15.80
6.9	4.34	5.66	19.17	25.0
7.2	2.71	7.29	9.29	25.0
7.5	1.53	8.47	4.52	25.0
7.8	0.80	9.20	2.18	25.0
8.1	0.41	9.59	1.07	25.0

* Stock 0.5 M Na_2HPO_4 and KH_2PO_4 are diluted 7.5-fold to bring the concentration to M/15. The M/15 solutions are then at once mixed in the indicated proportions.

TABLE III

*0.05 M Acetate Buffer Solutions**

pH at 38°	pK'$_{38°}$	Volumes to make 10 ml. buffer solution		Volumes to mix when larger fraction is 25 ml.	
		Na acetate	Acetic acid	Na acetate	Acetic acid
		ml.	*ml.*	*ml.*	*ml.*
4.9	4.667	6.31	3.69	25.0	14.62
5.2	4.655	7.78	2.22	25.0	7.13
5.5	4.649	8.76	1.24	25.0	3.52
5.8	4.645	9.35	0.65	25.0	1.75

* Stock 0.5 M solutions of acetate and acetic acid are diluted 10-fold and mixed in the indicated proportions.

Since the pH of most urines will fall within the range of brom-cresol purple, a preliminary test with this dye is made. If the pH is above or below the range of brom cresol purple, the fact can usually be observed by the eye as soon as the solutions are mixed, and a fresh cuvette is prepared with phenol red or brom cresol green.

Calculation

Optical density, D, is estimated as described for plasma pH (1).

The pH of the urine diluted 5.5-fold is estimated by interpolation of D on one of the standard curves. From this pH 0.09 is subtracted as a correction for the effect of the dilution in increasing the apparent pH of the urine.

This dilution effect was noted by Hastings, Sendroy, and Robson (2) in determinations by visual comparison, and has been confirmed for photometric measurements by data in the present paper, in which the photometrically measured pH of dilute urine is compared with the pH of undiluted urine measured by the glass electrode.

TABLE IV

Comparison of pH Measured in Undiluted Urine by Glass Electrode with pH of 5.5-Fold Diluted Urine Measured by Photometer

Urine No.	pH of undiluted urine by electrode (*a*)	pH of 5.5-fold diluted urine by photometer (*b*)	Difference (*b* − *a*)
1	6.20	6.27	0.07
2	5.53	5.64	0.11
3	6.32	6.39	0.07
4	5.28	5.36	0.08
5	5.08	5.12	0.04
6	5.18	5.25	0.07
7	7.04	7.14	0.10
8	5.12	5.24	0.12
9	5.32	5.42	0.10
10	5.23	5.32	0.09
11	6.86	6.93	0.07
12	6.52	6.64	0.12
13	5.27	5.38	0.11
Mean difference			0.09
Standard deviation of difference from mean difference			±0.024

EXPERIMENTAL

Handling Urine—Consistent results for successive determinations were obtained only when the urine was kept completely protected from air. It was kept in closed vessels over mercury (tube *H* of Fig. 6, p. 54 of Peters and Van Slyke (6)).

Photometric Measurements—Portions of 2 ml. of urine were forced by mercury pressure from the containing vessels up into 2 ml. pipettes, from which the urine was at once delivered into the 8 ml. portions of water plus

1 ml. of dye previously measured into the cuvettes. The urine in each cuvette was at once mixed with a footed rod with the water and dye, and was covered with oil and warmed to 38° for photometric measurement, as described for plasma pH determinations (1).

Determinations by Glass Electrode—The pH of the undiluted urines was measured in Michaelis' (7) modification of the glass electrode of MacInnes and Belcher (8). The electrode was standardized with M/15 phosphate buffer solution of pH 5.80, the standardization being checked immediately before and after each urine determination. From the vessel in which the urine was kept over mercury the urine was delivered directly into the cup of the electrode, and enough was drawn down into the capillary to establish liquid junction. The unused portion of urine remaining in the cup served to protect the urine below from loss of CO_2 during the measurement. No drift in potential was apparent during the time required to make the measurements.

Results

The results of simultaneous photometric and electrometric determinations on a series of human urines are given in Table IV. They indicate that, if the correction of 0.09 given under "Calculation" is applied, the photometric values thus corrected will usually be within ±0.05 pH unit of the electrometric values.

SUMMARY

A procedure is described for photometric measurement of the pH of urine. The standard deviation from pH values determined with the glass electrode was ±0.024 pH unit.

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PREPARATION OF RIBONUCLEOSIDES FROM SMALL AMOUNTS OF RIBONUCLEIC ACID

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The preparation of the various nitrogen-containing substances from ribonucleic acid (PNA) by the usual chemical methods is feasible only if considerable amounts of the nucleic acid are available. This is especially true for the pyrimidine nitrogen from cytosine and uracil. During tracer work with N^{15} on the nucleic acid turnover of liver nuclei and bacteria in this laboratory, it became desirable to isolate the different bases free from each other and from foreign nitrogen in amounts of a few mg. Partition chromatography on starch, as introduced by Elsden and Synge (1), was thought to give a convenient solution to our problem, especially since Vischer and Chargaff (2) had shown that the purine bases adenine and guanine can be separated on a paper chromatogram. In a recent publication (3) Hotchkiss has applied partition chromatography on paper to several nitrogen-containing compounds derived from nucleic acid. A method for the preparation and separation of the purine bases from PNA and desoxyribonucleic acid (DNA), which involves chromatography on a starch column, has been described by Edman, Hammarsten, Löw, and Reichard (4). The separation of the nucleosides from PNA on a starch column has been described in a preliminary report by the author (5).

The separation of the ribonucleosides was carried out because it was thought that their preparation in reasonable yield without conversion of cytosine to uracil would be easier than the preparation of the free pyrimidine bases. For the preparation of the ribonucleosides several different methods are available. The oldest one of Levene and Jacobs (6), which employs hydrolysis of the nucleic acid with dilute ammonia at high temperature, was not considered suitable because of the possible exchange of amino groups with ammonia. Levene (7) has also employed acid hydrolysis with 10 per cent sulfuric acid at 125° for the preparation of cytidine from cytidylic acid. He states that no conversion to uridine takes place. In experiments with small amounts of cytidylic acid it was found, however, that with different acid concentration and temperature a considerable amount of uridine was always formed. More recently Bredereck (8) has described a method for preparing the nucleosides by refluxing PNA for 4 days with a mixture of equal amounts of pyridine and water. By modifying his method so as to involve a 12 hour hydrolysis in a sealed tube at 115°, it was possible to avoid the formation of uridine from pure cytidylic acid. On the other hand the yield of the pyrimidine nucleosides, especially uridine, was low. By using higher temperature and a longer time for hydrolysis, practically all the cytidylic acid was converted to uridine. This method has been used in tracer experiments on normal and regenerating rat liver (9).

The best results were obtained by enzymatic hydrolysis of the ribonucleotides. Phosphatases which split off the phosphoric acid from nucleotides have been reported from different sources. Bredereck (10) uses an extract from emulsin for preparing the four ribonucleosides on a large scale. Hartmann and Bosshard (11) employ an enzyme preparation from potatoes for the preparation of adenosine. Both these crude enzymes and also alkaline phosphatase from calf intestine, prepared according to Schmidt and Thannhauser (12), have been tried and found to contain considerable desaminase activity. The acid prostate phosphatase of Schmidt, Cubiles, and Thannhauser (13), however, turned out to be satisfactory for the hydrolysis of ribomononucleotides to nucleosides. It could be shown (*a*) that the enzyme from prostate dephosphorylates mononucleotides at a very low concentration of nitrogen in the extract so that the addition of foreign nitrogen to the mononucleotides was negligible, (*b*) that the extract did not contain any desaminase activity, and (*c*) that the nucleoside-splitting activity of the extract was very low. Furthermore a method has been worked out for preparing a mixture of the four ribomononucleotides free from salts and foreign nitrogen after the separation of PNA and DNA according to Hammarsten (14).

EXPERIMENTAL

Enzyme Extract—An extract from enucleated hypertrophic human prostate was prepared according to Schmidt, Cubiles, and Thannhauser (13) by mixing the freshly enucleated prostate with 5 parts of a mixture of water and crushed ice in the Waring blendor. The mixture was allowed to stand in the ice box for about half an hour and then filtered by suction with the aid of Hyflo Super-Cel. The slightly turbid filtrate was used immediately, as the enzyme activity decreased rather rapidly, even when the extract was kept at −15°. The activity of various extracts was satisfactory. No attempt was made to purify the enzyme.

pH Optimum—To different samples of 38 mg. of pure adenylic acid in 10 ml. of water were added different amounts of 0.2 N sodium hydroxide, so that the pH ranged from 3.5 to 6.0. 1 ml. of the enzyme extract, diluted 1:500, was added to each sample and the amount of free phosphate determined after 12 hours digestion at 37° (Fig. 1). The value 4.3 found as pH optimum differs from that of 5.3 found by Schmidt, Cubiles, and Thannhauser. The discrepancy might be explained by the fact that the solutions were not buffered in the present experiments, as salts would interfere later on with the chromatography.

Enzyme Concentration—The extract from the gland had a nitrogen content of less than 1 mg. per ml. It was generally used in a dilution of 1:100. In this case less than 0.01 mg. of foreign nitrogen was added to a solution containing from 10 to 50 mg. of nucleotides. The amount of enzyme required for satisfactory hydrolysis was higher for the pyrimidine nucleotides than for the purine nucleotides (Fig. 2).

Digestion of Single Ribomononucleotide and Chromatography of Product—The following experiments were carried out in order to investigate the possible desaminating and nucleoside-splitting activity of the enzyme extract. About 10 mg. of the four pure ribomononucleotides were separately dissolved in 10 ml. of water. The solutions were neutralized with sodium hydroxide to a pH between 4 and 4.5. To each of the four solutions was added 1 ml. of the freshly prepared enzyme solution in a dilution of 1:100. The tests were incubated for 24 hours at 37°. The solutions were then evaporated to dryness on a steam bath. The residues were extracted successively with 2 ml. and 2 × 1 ml. of hot water-saturated butanol. The combined extracts from each residue were added to the top of four starch columns with a length of 220 mm. and an inner diameter of 23 mm. The chromatogram was developed and the effluents analyzed as described in a

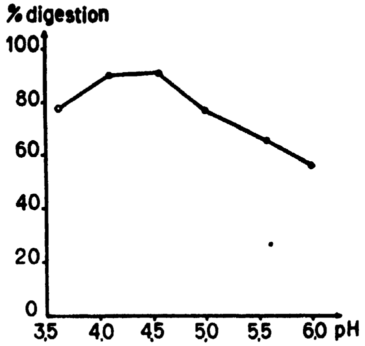

FIG. 1

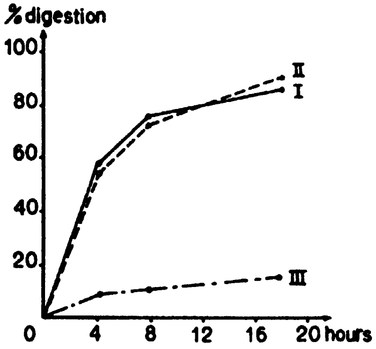

FIG. 2

FIG. 1. pH optimum for prostate phosphatase. 38 mg. of adenylic acid per 10 ml. of water titrated to a different pH with 0.2 N NaOH. 1 ml. of prostate extract, diluted 1:500, is added.

FIG. 2. Curve I, digestion of adenylic acid (10 mg. per 10 ml., pH 4.1) with 1 ml. of prostate extract, diluted 1:500; Curve II, digestion of cytidylic acid (10 mg. per 10 ml., pH 4.2) with a ml. of prostate extract, diluted 1:500; Curve III, same as Curve II, enzyme dilution 1:100.

previous paper (5). The results of the four different digestions and chromatograms are seen in Figs. 3 to 6. The light absorption at 2620 A in the effluent was measured in the Beckman photometer on 1 hour fractions and plotted against the volume of the effluent. It can be seen that, with the exception of adenylic acid, only one light-absorbing compound was obtained from each mononucleotide. The R values (15) for the different compounds, including the main compound in the case of adenylic acid, agree well,

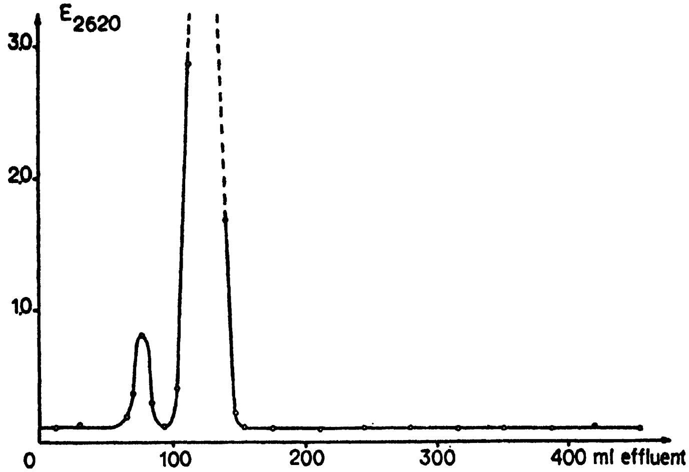

FIG. 3. Chromatography after digestion of 7.6 mg. of adenylic acid with prostate enzyme dilution 1:100. Yield of adenosine, 4.5 mg. (78 per cent).

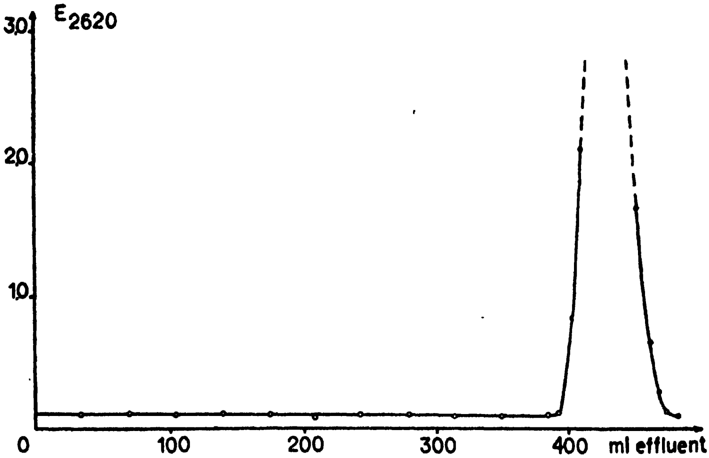

FIG. 4. Chromatography after digestion of 6.5 mg. of cytidylic acid with prostate enzyme diluted 1:100. Yield of cytidine, 3.5 mg. (yield 74 per cent).

within satisfactory limits, with those obtained from the pure corresponding nucleosides. Their identity was established in the following way. The light-absorbing fractions in each run (this means for adenylic acid only the light absorption corresponding to the second peak) were combined and the butanol evaporated *in vacuo*. The residues were dissolved in 10 ml. of 0.1 N HCl, and after proper dilution the light absorption measured between 2400 and 3100 A. The ratio $E_{max.}/\gamma$ N per ml. was determined

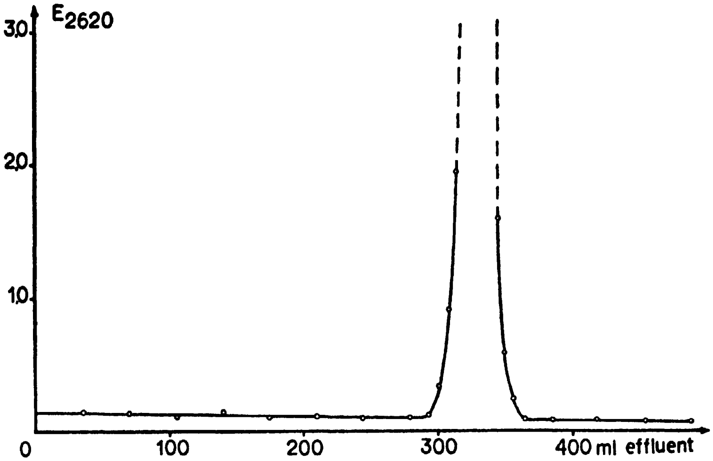

FIG. 5. Chromatography after digestion of 9.0 mg. of guanylic acid with prostate enzyme, diluted 1:100. Yield of guanosine, 4.9 mg. (yield 70 per cent).

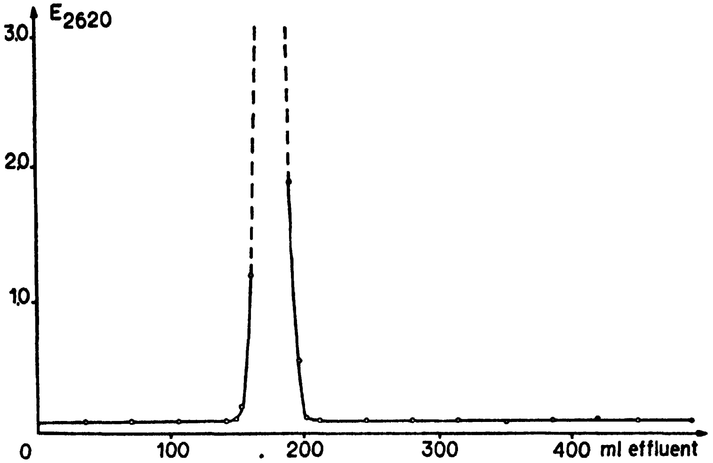

FIG. 6. Chromatography after digestion of 6.5 mg. of uridylic acid with prostate enzyme, diluted 1:100. Yield of uridine, 4.4 mg. (yield 89 per cent).

for each compound by analyzing an aliquot of the HCl solution for nitrogen. In this way it could be established that the corresponding nucleosides were formed from the four different mononucleotides (Table I) and that no desamination takes place.

In the case of adenylic acid one small extra peak appeared which migrated before the adenosine. In order to obtain larger amounts of this compound 10 mg. of adenylic acid were digested in the same way as above with the lower enzyme dilution of 1:25. The chromatogram showed the same principal features as in Fig. 3, but the first peak had now the same altitude as that of adenosine. The light absorption curve in 0.1 N HCl and the value $E_{\text{max.}}/\gamma$ N per ml. for the first peak showed good agreement with corresponding values for adenine (Table I). The definite proof that this compound really was adenine was established by running a mixture of adenine and adenosine on an identical starch column (Fig. 7).

TABLE I

Characteristic Values for Compounds Obtained after Digestion of Pure Mononucleotides, and Chromatography of Digestion Mixture As Compared with Standard Substances

	R value	$\frac{E_{\text{max.}}}{E_{2480}}$	$\frac{E_{2800}}{E_{2620}}$	$\frac{E_{2700}}{E_{2550}}$	$\frac{E_{\text{max.}}}{\gamma \text{ N per ml.}}$
1st compound from adenylic acid	1.19	1.39	3.90		0.184
Standard adenine	1.25	1.38	4.15		0.180
2nd compound from adenylic acid	0.75	1.23		0.66	0.205
Standard adenosine	0.80	1.25		0.69	0.196
Compound from cytidylic acid	0.21	3.72	1.58		0.290
Standard cytidine	0.21	3.55	1.51		0.295
Compound from guanylic acid	0.29	1.09		0.71	0.170
Standard guanosine	0.28	1.07		0.73	0.164
Compound from uridylic acid	0.48	1.53	0.34		0.345
Standard uridine	0.52	1.50	0.36		0.332

It may be that guanine too is formed during the digestion of guanylic acid, though because of its low solubility in butanol it would not interfere with the chromatogram. This might explain the relatively low yield of guanosine.

Preparation of Mixed Ribomononucleotides—The starting material was a PNA hydrolysate containing the different ribomononucleotides which was obtained by the preparation and separation of polynucleotides according to Hammarsten (14). The separation of the mixed polynucleotides with this method is carried out by hydrolysis of PNA with 0.1 N sodium hydroxide and precipitation of DNA with lanthanum nitrate and malonic acid. When the mixed ribonucleotides are to be isolated, hydrochloric acid is substituted for malonic acid in order to reach the pH range 2 to 2.5. In this case caution must be exercised to add enough HCl, because at a pH above 3 the ribomononucleotides start precipitating together with the DNA.

After the precipitation of DNA, the solution contains the ribomononucleotides and rather large amounts of salts and varying small amounts of denatured proteins and hydrolysis products from proteins. The purpose of the following procedure is to remove these contaminants as completely as possible before the digestion with the phosphatase and chromatography.

The mononucleotides were precipitated by adding to the hydrolysate 0.2 volume of a saturated solution of mercuric nitrate in water. The mononucleotides were allowed to precipitate for 24 hours at +2°. The precipitate was centrifuged and washed three times with 10 ml. of water. After the last washing, the precipitate was suspended in 20 to 30 ml. of water and decomposed with hydrogen sulfide. The excess hydrogen sulfide was boiled out over a free flame and the hot solution centrifuged. The mercuric sulfide was washed twice with 10 ml. of boiling water and the washings added to the main solution. The combined solutions were evaporated on a steam bath to about 15 ml. There is now usually a more or less notable opalescence, owing to the presence of traces of lanthanum compounds.

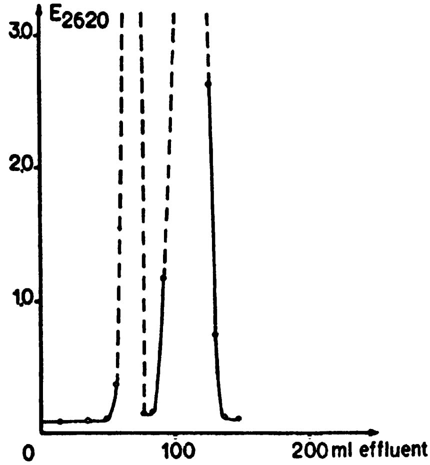

FIG. 7. Chromatography of 2.4 mg. of pure adenine and 3.6 mg. of pure adenosine

The solution of the mononucleotides was electrodialyzed according to Theorell and Åkesson (16). In this way the last traces of lanthanum, which interfere with the digestion, were removed. If any amino acids were left, only the acid ones would move to the anode compartment together with the mononucleotides. The acid amino acids have very low *R* values in butanol-water and because of this would be removed during the chromatography. The mononucleotides moved quantitatively into the anode compartment, as indicated by model experiments with pure substances. If the mononucleotides are obtained from a hydrolysate of PNA as outlined above, however, then at the end of the dialysis some light-absorbing material also appears in the cathode compartment, though it scarcely exceeds 5 to 10 per cent of the total light absorption. The cathode solution could be precipitated by silver nitrate at pH 2 (17), indicating that the light absorption is caused by the presence of free purine bases which have been split off during the preparation.

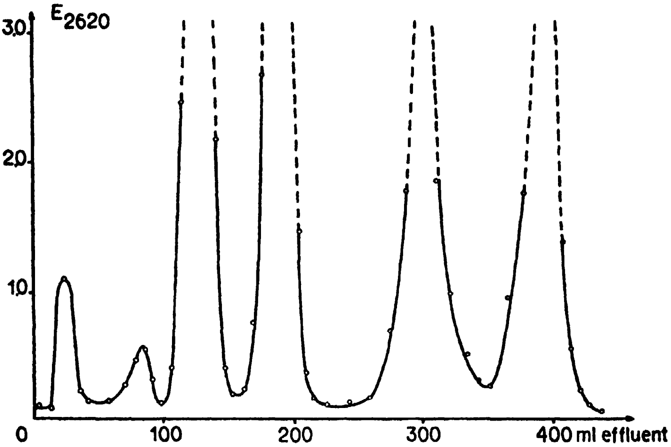

FIG. 8. Chromatography in model experiment with all four ribomononucleotides. The effluent corresponding to the peak before the adenine was slightly yellowish; its light absorption was not of the pyrimidine type.

TABLE II

Model Experiment on Mixture of Four Ribomononucleotides

Acid	Nucleotide in starting material	Yield of nucleoside	Yield	$\frac{E_{\text{max.}}}{\gamma \text{ N per ml. nucleoside}}$
	mg.	*mg.*	*per cent*	
Adenylic	10.1	5.2	66	0.205
Cytidylic	6.9	2.9	56	0.303
Uridylic	6.6	3.9	79	0.331
Guanylic	9.5	3.5	47	0.174

After the end of the electrodialysis, the solution from the anode compartment containing the ribomononucleotides was neutralized with 0.5 N sodium hydroxide to pH 4 to 4.5 and subjected to digestion.

Model Experiment on Mixture of Four Ribomononucleotides—In order to determine the over-all yield of the whole procedure, about 10 mg. each of pure mononucleotides were dissolved in 10 ml. of NaOH, lanthanum and HCl were added after 2 hours on a steam bath, and the whole method of preparation as described above was carried out. The result of the chromatography after digestion can be seen in Fig. 8 and Table II.

SUMMARY

A phosphatase-containing extract from prostate was investigated with respect to the splitting of ribomononucleotides to the corresponding nucleosides. Good yield of nucleosides was obtained and little or no deamination took place.

The extract was used for obtaining nucleosides from PNA, separation being achieved chromatographically.

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ON THE NITROGEN TURNOVER IN PURINES FROM POLYNUCLEOTIDES DETERMINED WITH GLYCINE N^{15}

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The work of Brown *et al.* (1) with N^{15}-containing purines has shown that, of the naturally occurring purines, only adenine can be utilized directly for the synthesis of polynucleotides. When isotopic adenine was fed to the rat, part of it was recovered in rather low dilution in the adenine and in a somewhat higher dilution in the guanine of the polynucleotides from the mixed internal organs. By separating ribonucleic acid (PNA) from desoxyribonucleic acid (DNA), Brown, Petermann, and Furst (2) showed that the adenine had almost entirely entered the PNA fraction. Though these experiments prove that adenine administered in the food can act as a precursor not only of PNA adenine but also of PNA guanine, it does not follow that PNA guanine is formed ordinarily from adenine. Barnes and Schoenheimer (3), in their experiments with N^{15}-containing ammonium citrate, found in the pigeon a higher content of isotope in guanine than in adenine. They were, however, able to show that this was due to a higher N^{15} content in the amino group of guanine than in that of adenine. In experiments carried out in this laboratory (4) with N^{15}-glycine as a precursor for nucleic acid purines and pyrimidines in the hepatectomized and normal rat, it was found that the guanine in both the PNA and DNA fractions had about twice the isotope content of adenine. This finding makes it somewhat improbable that guanine could have been synthesized by way of adenine. The present investigation was carried out in order to settle this question. The experiments were performed with cytoplasm from regenerating liver and intestine from normal rat which had been left over from a previous experiment (4). The aminopurines were isolated from the PNA of cytoplasm and the PNA and DNA from intestine. Part of them was analyzed for N^{15} and the rest subjected to degradation to the corresponding hydroxypurines and these, too, were analyzed for their isotope content. A new method for the preparation of xanthine and hypoxanthine from the aminopurines with chromatography on a starch column was developed for that purpose. With this method as little as 2 mg. of aminopurine can be degraded to the corresponding hydroxypurine. The results of the isotope analyses for the different purines are summarized in Tables I and II.

The figures demonstrate that the isotope content is always higher in guanine than in adenine. The amino group of adenine always has a low isotope content, as indicated by the higher value of hypoxanthine compared with the corresponding adenine. The amino group of guanine in the PNA fractions has a relatively high isotope content, while in the DNA from intestine this is low. It can be seen that a reversal takes place for the two purines from the PNA of intestine after deamination; though guanine has a higher isotope content than adenine, the guanine nucleus, as indicated by the results for xanthine, has a lower isotope content than the adenine nucleus, as indicated by the data for hypoxanthine. This is, however, not the case for PNA purines from regenerating liver cytoplasm or for DNA purines from intestine.

TABLE I

Aminopurines and Corresponding Hydroxypurines from Intestine of Rat

The ratio $E_{\text{max.}}/\gamma$ N per ml., which is a measure of purity with respect to foreign nitrogen, is for the pure compounds, adenine 0.180, hypoxanthine 0.197, guanine 0.161, and xanthine 0.170.

	PNA		DNA	
	Excess N^{15}	$\frac{E_{\text{max.}}}{\gamma \text{ N per ml.}}$	Excess N^{15}	$\frac{E_{\text{max.}}}{\gamma \text{ N per ml.}}$
	atom per cent		*atom per cent*	
Adenine	0.46	0.171	0.27	0.169
Hypoxanthine	0.54	0.191	0.33	0.192
NH_2 group (calculated)	0.12		0.06	
Guanine	0.51	0.165	0.48	0.161
Xanthine	0.43	0.165	0.59	0.169
NH_2 group (calculated)	0.83		0.04	

TABLE II

Aminopurines and Corresponding Hydroxypurines from Regenerating Liver Cytoplasm of Rat

	Excess N^{15}	$E_{\text{max.}}/\gamma$ N per ml.
	atom per cent	
Adenine	0.43	0.178
Hypoxanthine	0.51	0.190
NH_2 group (calculated)	0.11	
Guanine	0.97	0.158
Xanthine	0.97	0.167
NH_2 group (calculated)	0.97	

EXPERIMENTAL

Administration of N^{15}-Glycine—Organs from a previous experiment (4) were used. The rats had been given two subcutaneous injections of 50 mg. of glycine per 100 gm. of body weight with an interval of 6 hours between doses. The glycine contained 32 atom per cent excess of N^{15}. 6 hours after the last injection the rats were killed and the various organs were immediately put into a large excess of absolute alcohol. They were homogenized with alcohol in the Waring blendor, filtered, and dried with alcohol and ether. The hepatectomized rats received the same treatment starting 16 hours after the operation. In this case the livers were put into cold citric acid, and cell nuclei and cytoplasm were separated immediately after death 28 hours from the time of operation. The details are described in a previous publication (4).

Isolation of Adenine and Guanine—The procedure was mainly that of Edman, Hammarsten, Löw, and Reichard (5), though with some modification. The purines were obtained, after separation by the method of Hammarsten (6) of the PNA and DNA, by hydrolysis with N sulfuric acid for 1 hour at 100°. The excess sulfate was precipitated by adding freshly prepared hot solution of barium hydroxide, saturated at boiling temperature to pH 2. The precipitate of barium sulfate was centrifuged immediately and extracted twice with 5 ml. of hot water. The combined supernatants were precipitated with 0.2 volume of a 1 M solution of silver nitrate, as described by Kerr and Seraidarian (7). The precipitate of the silver purines was centrifuged, washed twice with 10 ml. of 0.05 M silver nitrate, twice with alcohol, and once with ether. The dry silver purines were decomposed with 5 ml. + 3 ml. + 3 ml. of 0.5 N HCl, according to the method of Kerr and Seraidarian (7). The HCl solution was evaporated to dryness *in vacuo* and the rest of the HCl carefully removed by repeated evaporation.

The residue was dissolved by warming in 1.0 ml. of water containing 0.1 m.eq. of sodium hydroxide. To the hot water solution, 10 ml. of dry butanol were added and the aqueous solution of the purines dissolved in the butanol by warming and gentle shaking. The butanol-water solution was added to the top of a starch column and the chromatogram was run and analyzed as previously described (5), the only difference being that the solvent used for developing the chromatogram was butanol-water (135 ml. of water + 865 ml. of butanol) and did not contain any glycol methyl ether.

Deamination of Adenine or Guanine to Corresponding Hydroxypurines—The method is a modification of the first steps of those of Strecker (8) and Kossel (9) to suit the degradation of small amounts of aminopurines. The aminopurine (2 to 8 mg.) was obtained in dry form after chromatography by evaporating *in vacuo* the butanol of the combined fractions containing the same purine base. It was transferred to a 40 ml. centrifuge tube with about 2.5 ml. of N H_2SO_4. About 0.5 ml. of this solution was set aside for establishing the presence or absence of foreign nitrogen by the ratio $E_{max.}/\gamma\ N$ per ml. (5) after proper dilution with N HCl and for isotope analysis. The volume of the rest of the solution was reduced to one-fourth, so that the sulfuric acid became 4 N. To this solution the necessary amount of sodium nitrite (8 mg. of nitrite per 1 mg. of aminopurine) was added, dissolved in 0.2 ml. of water. This was carried out slowly over a period of 10 minutes with continuous stirring. During this time the centrifuge tube containing the solution was placed in a boiling water bath and allowed to stand there for another 5 minutes after the last addition of the nitrite. Since the solution becomes more concentrated by evaporation during the whole procedure, 1 drop of water should be added now and then in order to keep the volume constant. The solution was then diluted with 15 to 20 ml. of water and precipitated hot with barium hydroxide to pH 2. The further procedure was the same as that described for the preparation of adenine and guanine; *i.e.*, precipitation of the supernatant with silver, washing of the precipitate, decomposition with HCl, and evaporation of the acid. It was found adequate to reprecipitate the hydroxypurine once with silver nitrate. This was done in the usual way after dissolving the residue after the careful removal of the HCl in about 10 ml. of 0.01 N sulfuric acid. The free hydroxypurines were prepared from the silver purines as described above.

The hydroxypurines prepared in this way usually contain very little, if any, aminopurines. The highest contamination found was about 10 per cent. In order to remove the aminopurines, and as a further step of purification, the hydroxypurines were subjected to chromatography on a starch column.

Chromatographic Model Experiment with Adenine and Hypoxanthine—About 2 mg. of each of the purines were dissolved in butanol-water with the aid of NaOH in the same way as described for the adenine-guanine chromatography. They were put onto a starch column (length 100 mm., diameter 36 mm.), the chromatogram was run, and the purines localized in the effluent by measuring their light absorption in the ultraviolet (Fig. 1). Fractions containing the same purine were combined, the butanol evaporated *in vacuo*, and the residue dissolved in 10 ml. of N HCl. By taking an aliquot of the solution for nitrogen determination and determining the light absorption after proper dilution with N HCl, the ratio $E_{max.}/\ \gamma$ N per ml. was determined for each purine and compared with the same ratio for the pure purine. This ratio was, for pure hypoxanthine, 0.197 ($E_{max.}$ at 2480 A) and for adenine, 0.180 ($E_{max.}$ at 2620 A).

Chromatographic Model Experiment with Guanine and Xanthine—Fig. 2 shows a chromatogram for these two bases run under about the same conditions as described above for adenine and hypoxanthine. The length of column in this experiment was 122 mm., however. Another difference is that in chromatographing solutions containing xanthine, as little alkali as possible should be used to dissolve the purines in butanol-water. If excess alkali is used, the resolving power of the column declines and, furthermore, the yield of xanthine drops. In the experiment of Fig. 2, 0.03 m.eq. of NaOH was used for a total of 4 mg. of the bases.

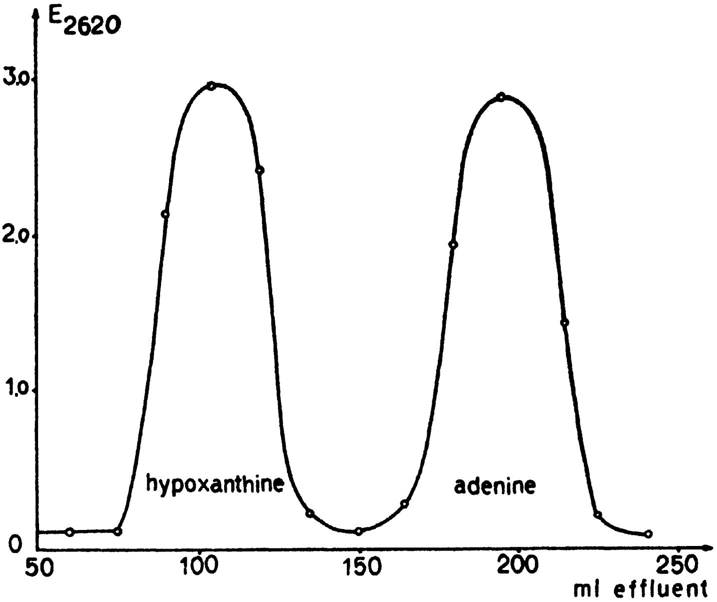

FIG. 1. Separation of 1.8 mg. of pure adenine and 2.4 mg. of pure hypoxanthine on a starch column. Length of the column, 97 mm., inner diameter 36 mm. Fractions (about 15 ml.) were collected for each ½ hour. The *R* values, according to Martin and Synge (10), were adenine 0.94 and hypoxanthine 0.49. Yield of adenine 96 per cent, of hypoxanthine 87 per cent.

As can be seen from Fig. 2, a complete separation of xanthine from guanine has not been achieved in this experiment. The separation would, however, have been practically complete if the amount of guanine had been small as compared with that of xanthine, as is the case of a degradation experiment. Complete separation can also be achieved with larger amounts of guanine if a longer column is used for the chromatogram. However, this does not usually improve the total yield of pure xanthine. The reason is that part of the xanthine is lost during the chromatography by sticking in the column. These losses, which for a column of 120 mm. are not more than at the most 20 per cent, increase rather rapidly with the length of the column.

If part of the guanine appears in the same fractions as part of the xanthine, these mixed fractions can easily be distinguished by determining the ratio of the light absorption at 2620 and 2480 A for every fraction. This is indicated by the dash line in Fig. 2. At first, when only xanthine is emerging from the column, the ratio is constant and lies at about 2.20. In the mixed fractions the ratio rapidly declines, the more the xanthine is contaminated with guanine, and finally reaches a constant value of about 0.75 when the fractions consist of pure guanine. A good yield of the bases is still obtained even when the mixed fractions are discarded. Their purity with respect to foreign nitrogen is established in the usual way. The ratio $E_{\text{max.}}/\gamma$ N per ml. is, for guanine, 0.161 ($E_{\text{max.}}$ at 2480 A), and for xanthine 0.170 ($E_{\text{max.}}$ at 2600 A).

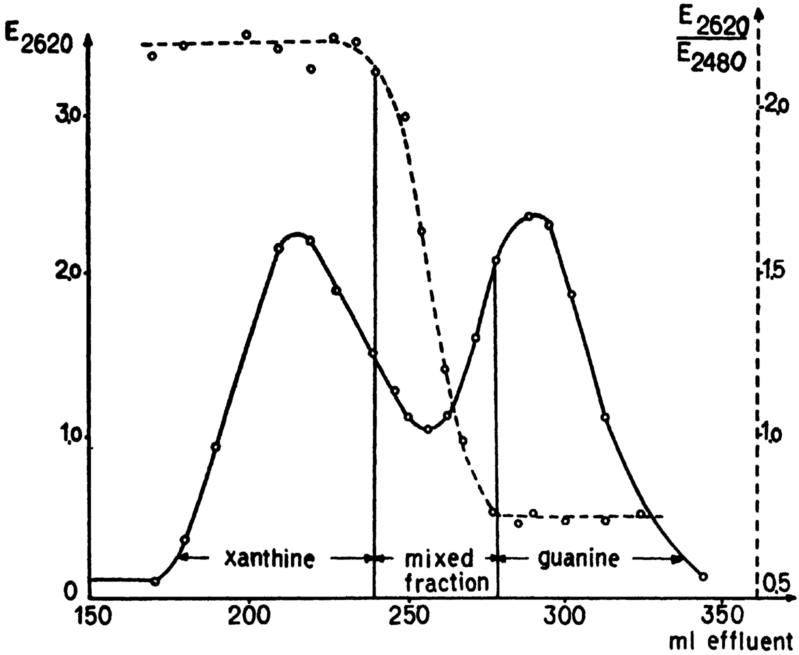

FIG. 2. Separation of 2.3 mg. of pure xanthine and 1.7 mg. of pure guanine on a starch column. Length of the column, 122 mm., inner diameter 36 mm. The half hourly fractions were collected. *R* values (10), xanthine 0.58, guanine 0.43. The dash line gives the ratio E_{2620}/E_{2480} and indicates where the xanthine starts to become contaminated with guanine. Yield of pure xanthine 78 per cent, pure guanine 73 per cent.

The over-all yield for the deamination of the aminopurines and chromatography of the hydroxypurines was established with pure adenine and guanine. For both cases it was found to be 60 to 70 per cent when starting with 2 to 8 mg. of aminopurine.

DISCUSSION

Glycine has been shown by Abrams, Hammarsten, and Shemin (11) to act as a precursor for the nitrogen in the 7 position of nucleic acid purines. According to the views expressed by Brown *et al.* (1) adenine would be synthesized before guanine, which would subsequently be synthesized from the adenine nucleus. That this *can* be the case has been shown by Brown's feeding experiments with isotopic adenine. If this theory corresponds to a general pathway for the synthesis of nucleic acid purines, one would expect it to hold true irrespective of the nature of the isotopic precursor for the purines. One would expect furthermore that the isotope content of adenine would not be lower than that of guanine. This was also the case in Brown's experiments. The experiments of Hammarsten and coworkers (4) on normal and regenerating liver show, however, that in both PNA and DNA the isotope content of guanine is much higher than that of adenine. These findings could be brought into line with Brown's theory if it is assumed that the amino group in guanine has a much higher N^{15} content than the rest of the molecule. At this stage no definite answer could be given to that question as no method for degradation of small amounts of aminopurines was available to us.

The present experiments show that in regenerating liver cytoplasm the main pathway for the synthesis of PNA guanine, with glycine as a precursor, does not proceed via adenine. In accordance with the finding of Barnes and Schoenheimer (3), who used ammonia as a precursor, it was found that the amino group of guanine has a higher turnover rate than that of adenine. Its isotope content, however, is not high enough to suggest that the remaining guanine residue could have been formed from adenine.

The findings with respect to the PNA from intestine would agree rather well with Brown's theory. There, the difference between the isotope content of guanine and adenine is much smaller and after deamination the relations are inversed, so that the isotope content in hypoxanthine is higher than that of xanthine.

The DNA purines from normal intestine differ from the PNA purines from the same organ in that the amino groups of both adenine and guanine have a relatively low isotope content. As the guanine has a much higher isotope content than the adenine, this proves that in this case the synthesis of guanine has not proceeded via adenine. A difference between PNA purines and DNA purines has been noted earlier by Brown *et al.* (2).

In feeding experiments on rats with N^{15}-adenine, the adenine from the PNA of the mixed internal organs contained about 30 times as much isotope as the adenine from the corresponding DNA. The authors thought the reason for this to be a much lower turnover of the purines in DNA as compared with the purines from PNA. Furthermore, it was assumed that the difference in the turnover ratio DNA to PNA between adenine and phosphorus (12) might indicate that "some portion of the phosphate moieties of a nucleic acid may be exchanged without the purines of the C—N skeleton being affected." In the present investigation the values for the turnover rates of the purines match fairly well with those obtained by Hammarsten and Hevesy (12) for phosphorus. In their experiment on intestine of the rat the turnover ratio DNA to PNA for phosphorus was 1.7 as compared with the value 1.7 for adenine and 1.1 for guanine in the present investigation. Because of this, the finding of Brown *et al.* is explained better perhaps on the hypothesis that adenine cannot be utilized for the synthesis of purines in DNA.

The results obtained in the present investigation clearly indicate the necessity for separating the polynucleotides into DNA and PNA in isotope experiments. Furthermore, they show that there might be a difference in the purine turnover in different organs.

SUMMARY

A method for obtaining the corresponding hydroxypurines from small amounts (2 to 8 mg.) of adenine and guanine has been developed.

Intestine and regenerating liver cytoplasm from a previous experiment (4) in which N^{15}-glycine had been injected into rats has been worked up for aminopurines of PNA and DNA. These were degraded to the corresponding hydroxypurines.

Evidence has been obtained that the main part of guanine in PNA from regenerating liver cytoplasm and from DNA in intestine is not synthesized via adenine. In the case of PNA from intestine this possibility could not be excluded.

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THE USE OF ISOTOPIC CARBON IN A STUDY OF THE METABOLSIM OF ANTHRANILIC ACID IN NEUROSPORA*

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The finding by Tatum, Bonner, and Beadle (1), that the *tryptophanless Neurospora* mutant strain 10575 accumulates anthranilic acid, which in turn can be utilized for growth of strain 40008, has provided evidence that anthranilic acid is a biochemical precursor of tryptophan in this organism. It has been further established that indole is an intermediate in this conversion (2–5).

More recent work with a number of mutants of *Neurospora* (6–8) has established that tryptophan is a biochemical precursor to niacin with kynurenine and hydroxyanthranilic acid as intermediates. The accumulated evidence has indicated the existence in the mold of the following series of reactions:

→ Anthranilic acid → indole → tryptophan → kynurenine → 3-hydroxyanthranilic acid → nicotinic acid

In the light of this evidence the present work was undertaken to trace the carbon in the carboxyl group of anthranilic acid in order to estimate its contribution as a structural unit in the formation of niacin and tryptophan. The organism chosen for this investigation was a biochemical mutant strain of *Neurospora* designated as strain 40008. This mutant utilizes anthranilic acid, indole, or tryptophan for growth.

The mutant was grown in the presence of anthranilic acid containing C^{14} in the carboxyl group. Niacin and tryptophan were isolated from the mold mycelium and tested for radioactivity.

EXPERIMENTAL

Growth of Mold—Neurospora mutant 40008 was grown under forced aeration for 6 days at room temperature in 10 liters of minimal medium

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(9) to which a supplement of 16.7 mg. of active anthranilic acid was added. A trap containing 40 per cent NaOH was connected to the system to absorb any carbon dioxide evolved during growth of the mold. At the end of the growth period the mold was filtered off, and the moist mycelium was continuously extracted with 300 ml. of acetone for 20 hours. The dried residual mycelium weighed 3.7 gm.

Isolation of Niacin—The acetone extract of the mycelium was taken to dryness, redissolved in 50 ml. of water, and filtered. It contained 700 γ of nicotinic acid or its amide.[1] After removing a 4 per cent aliquot for further study, 4 gm. of anhydrous barium hydroxide were added to the main portion of the extract, and it was heated at 90° for 40 minutes, titrated to pH 6.5 with 2 N H_2SO_4, and filtered. The filtrate was taken to pH 4.2 with concentrated HCl and shaken with 1 gm. of norit A. This was filtered off and washed well with water.

The charcoal was eluted with two 50 ml. portions of hot 4 per cent aniline in water. The combined eluates were extracted with ether to remove the aniline and taken to dryness. The residue was taken up in 13.5 ml. of hot absolute alcohol and filtered. After readjusting the volume of the filtrate to 13.5 ml., a 1.5 ml. portion was taken for bioassay and investigation with paper chromatography. The total filtrate contained, according to bioassay, 300 γ of nicotinic acid. This material was established as nicotinic acid by comparing its movement on an ascending paper chromatogram with that of an authentic sample of nicotinic acid. Butanol saturated with water was the solvent used for developing the chromatograms.

40 mg. of nicotinic acid were added to 12 ml. of the original 13.5 ml. of filtrate containing the isolated nicotinic acid. The nicotinic acid which precipitated from a concentrated aqueous solution was recrystallized from a 1:4 mixture of acetic acid and benzene. The isolated nicotinic acid melted at 229–232° and gave the same ultraviolet absorption spectrum as an authentic sample of the same acid.

Isolation of Tryptophan—10 mg. of pepsin (1:10,000 potency) were added to the 3.7 gm. of ground dry mycelium suspended in 50 ml. of 0.1 N H_2SO_4. The mixture was covered with toluene and incubated 21 hours at 37°. At the end of the first digestion 3 gm. of $K_2HPO_4 \cdot 12H_2O$ were added and the pH was adjusted to 8.4. The mixture was incubated at 40° for 72 hours in the presence of 50 mg. of trypsin (1:300 potency). The undigested suspended material was filtered off and washed with water. The combined filtrate and washings, diluted to 100 ml. with water, contained 13.2 mg. of tryptophan. The identity of this tryptophan was confirmed by comparing its position on an ascending paper chromatogram with that of an authentic tryptophan sample. Water-saturated butanol was used as the solvent for the chromatogram.

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[1] *Lactobacillus arabinosus* strain 17-5 was used to determine nicotinic acid and tryptophan.

To 20 ml. of the original 100 ml. of enzyme digest were added 20 mg. of stock L-tryptophan and 3 ml. of 50 per cent sulfuric acid. The mercury salt of tryptophan was precipitated by the addition of 2 gm. of $HgSO_4$ in 10 ml. of 6 per cent sulfuric acid. The mixture was allowed to stand 48 hours, at the end of which time the yellow precipitate was filtered off, washed with 5 per cent sulfuric acid, and finally with water. The moist mercury precipitate was suspended in 8 ml. of water and made alkaline to phenolphthalein with a concentrated barium hydroxide solution. A rapid stream of hydrogen sulfide was passed into this alkaline solution to precipitate the mercury as its sulfide. This was filtered off and washed with water. Traces of barium ion were removed by the addition of a drop of dilute sulfuric acid followed by filtration. The filtrate was taken to a pH of 5.9 with sodium carbonate, concentrated to about 5 ml. under a vacuum, and continuously extracted for 12 hours with 75 ml. of butanol. After adjusting the butanol extract back to 75 ml., a 5 ml. aliquot was taken for bioassay. The total butanol solution contained 9.4 mg. of tryptophan. The bulk of the extracted tryptophan contained in 70 ml. of butanol was taken to dryness. 40 mg. of stock tryptophan were added to the residue

TABLE I

Distribution of Radioactivity in Mold Culture Components

Sample No.	Component	Total counts per sec.	Activity* per component	Activity* accounted for
			per cent	*per cent*
1	Culture fluid after removal of mycelium	57,500	7.2	7.2
2	Carbon dioxide trapped during growth of mold	739,652	92.3	92.3
3	Acetone-extracted material from mycelium	6,500	0.8	0.8
4	Isolated nicotinic acid	0.0	0.0	
5	Mycelium after acetone extraction	19,686	2.4	
6	Water-insoluble material after enzymatic digestion of mycelium	2,689	0.3	0.3
7	Water-soluble material after enzymatic digestion of mycelium	11,860	1.5	1.5
8	Isolated tryptophan	0.0	0.0	
Total				102.1

* Based on assay of original anthranilic acid, 48,000 counts per second per mg. Total activity used = 16.7 × 48,000 = 801,600 counts per second.

and the whole was taken up in 4 ml. of water. The volume was reduced to 1.5 ml., from which the tryptophan did not precipitate, even though the solubility of the solute in that volume of water at room temperature had been greatly exceeded. Small amounts of discolored material which precipitated were filtered off. The filtrate was taken to dryness and the residue crystallized twice from 60 per cent ethanol, m.p. 273-276°. The melting point of an authentic sample of tryptophan taken simultaneously was 275-278°. The ultraviolet absorption spectrum of the isolated material was identical with that of the authentic tryptophan used for isotopic dilution.

Distribution of Radioactivity—A general summary of the distribution of radioactivity in the various components of the mold culture is given in Table I.

DISCUSSION

The absence of radioactivity in the isolated niacin and tryptophan shows that, if the carbon in the side chain of anthranilic acid was incorporated as a structural unit in the formation of these two substances in *Neurospora*, it was lost during further metabolism involving these substances. The organism used for determining the tryptophan content of the mold digest does not differentiate between anthranilic acid, indole, tryptophan, and possibly peptides of tryptophan. This ambiguity was overcome by the use of paper chromatography. Not only was the isolated tryptophan identified by its position on a chromatogram, but a separation of biological activity and radioactivity was also effected by the same technique. The problem of differentiating nicotinic acid from its amide prior to dilution was also resolved by paper chromatography. Here again, a complete separation of radioactivity from biological activity was possible.

The presence of large quantities of isotopic carbon in the carbon dioxide evolved during the growth of the mold indicates that a major portion of the carboxyl group in anthranilic acid finds its way into this substance during growth of the mold.

SUMMARY

1. Anthranilic acid containing C^{14} in the side chain was given to a *Neurospora* mutant form which required either anthranilic acid, indole, or tryptophan as a supplement for growth. Nicotinic acid and tryptophan isolated from the mold tissue were found to contain no detectable quantity of the isotopic carbon.

2. A large part of the isotopic carbon was accounted for in the carbon dioxide evolved during the growth of the mold.

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CARNOSINASE: AN ENZYME OF SWINE KIDNEY*

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Since it was shown in a previous report (1) that certain peptidases were capable of hydrolyzing peptides containing β-alanine, the present investigation was undertaken to determine the possible metabolic fate of the naturally occurring peptide, carnosine. Although this substance, β-alanyl-L-histidine, has long been known and is one of the most abundant of the non-protein nitrogenous constituents of muscle, little is known regarding its metabolism or function (2). However, in the past few years it has been observed (3) that crude preparations of certain tissues, particularly kidney, spleen, and liver, can hydrolyze carnosine, but this peptide is not split by muscle tissue; nor is the autolysis of muscle affected by the addition of carnosine (4). This work suggests that the first step in the metabolism of carnosine is its hydrolysis into the constituent amino acids. This is also indicated by the work of du Vigneaud, Sifferd, and Irving (5), who found that the histidine of carnosine is available for the growth of animals on a histidine-deficient diet.

We have utilized the excellent procedure of Sifferd and du Vigneaud (6) for the synthesis of carnosine. It has been found that certain tissues can hydrolyze this peptide, and we have named the responsible enzyme carnosinase, since its properties are distinct from those of any previously described protease. The carnosinase of hog kidney has been partially purified and has been shown by inhibition and activation studies to be a metal-enzyme like other exopeptidases. The specificity of this enzyme has also been studied by its action on synthetic substrates.

Distribution and Preparation of Enzyme

Carnosine is hydrolyzed by certain tissues of the rat. Since the hydrolysis follows zero order kinetics, the rates have been calculated as K^0, per cent hydrolysis per minute. The relative activities, calculated per gm. of fresh rat tissue, were for kidney $K^0 = 1.2$, liver 1.5, spleen 0.7, and skeletal and heart muscle 0.0. A desiccated preparation of hog kidney (Viobin

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Corporation) was quite active. However, fresh frozen hog kidney gave superior activity and yields. This tissue was used in making two preparations of carnosinase: Preparation A, which contained no added metal ions and was useful for activation studies with metals and for testing the effect of inhibiting agents, and Preparation B, which was derived from Preparation A by treatment with Mn^{++} and was the most active preparation obtained.

Preparation A—Fresh frozen hog kidney (400 gm.) was ground in a mechanical meat grinder before the tissue had completely thawed. The grinder was rinsed with 1600 cc. of water which were used to extract the enzyme. Larger volumes of water or saline solutions gave less satisfactory extractions. After standing for 10 to 15 minutes, the mixture was centrifuged for 45 minutes at 2500 R.P.M. in the cold room. The upper fatty layer was then aspirated and the turbid enzyme solution was decanted from the insoluble residue, which was discarded. This enzyme solution (1300 cc.) had a protein N content of 1.3 mg. per cc., and a proteolytic coefficient, C^0 of 0.38. (The zero order proteolytic coefficient, C^0, is equal to K^0 per mg. of protein N per cc. of the reaction mixture.) The yield of this extraction was $K^0 = 0.5$ per gm. of moist tissue.

One-tenth volume (130 cc.) of toluene was added to the enzyme solution and the mixture was heated in a water bath at 55° for 15 minutes. The mixture was quickly cooled to 0°, and 179 gm. of ammonium sulfate (144 gm. per liter) were added to achieve 25 per cent saturation. The resulting precipitate was collected on fluted filter paper and discarded. (This and the succeeding steps in the procedure were performed at 2°.) The clear red filtrate (1100 cc.) was brought to 55 per cent saturation with 211 gm. of ammonium sulfate (192 gm. per liter) and was again filtered through fluted paper. The red filtrate was discarded. After thorough dialysis of the precipitate, the fine suspension containing the enzyme was centrifuged. About one-third of the activity remained in the clear, brownish supernatant; the remainder was present in the residue. The residue was extracted with 20 cc. of 0.15 M sodium chloride and centrifuged; the insoluble, inactive material was discarded. The slightly opalescent saline extract (20 cc.) is Preparation A. It contained 3.9 mg. of protein N per cc., and $C^0 = 0.86$. This represents a purification of 2.3 times and a yield of 13 per cent based on the crude water extract. The soluble fraction obtained after dialysis was less pure ($C^0 = 0.35$) and represented 7 per cent of the original activity.

The very limited solubility of the enzyme in water suggests that it has the properties of a globulin. About 20 per cent of the carnosinase activity is lost after standing for 8 days at 2°.

Preparation B—Further purification of the enzyme was achieved by the addition to 7.5 cc. of Preparation A of sufficient manganous chloride to make the solution 0.01 M, of enough veronal (diethyl barbiturate) buffer to make the solution 0.01 M, and of 0.1 M sodium hydroxide to bring the pH to 7.9 to 8.1. This mixture was then heated at 40° for 1 hour, whereupon a precipitate formed. The removal of this precipitate by centrifugation left a clear and almost colorless solution (14 cc.) which contained all of the activity. This is Preparation B; it contained 1.9 mg. of protein N per cc., and $C^0 = 5.6$. The true yield in this purification cannot be ascertained because of the activation produced by manganese (see below). However, the apparent purification was 14.7 times compared with the proteolytic coefficient of the crude water extract.

TABLE I

Relation of Hydrolysis Rate to Carnosinase Concentration

Appropriate dilutions of Preparation B were made just before use with 0.01 M $MnCl_2$ in 0.01 M veronal, pH 8.0. Hydrolysis was measured at 40° in 0.04 M veronal at a pH of 8.0 ± 0.2; the substrate concentration was 0.05 M.

Enzyme concentration	Time	Hydrolysis	K^0	Average, C^0
protein N per cc.	*hrs.*	*per cent*		
14.5			0.072	4.9
29	1	10	0.16	
	3	26	0.15	
	4	37	0.15	
	5	42	0.14	
	7	56	0.13	
	9.5	71	0.13	5.0
54			0.26	5.1
109			0.61	5.5
181			0.98	5.3
181	0.75	44	0.98	
	1	55	0.92	
	1.25	75	1.00	5.3

Properties of Carnosinase

Kinetics—The hydrolysis of carnosine by swine kidney preparations has been found in all cases to follow the kinetics of a zero order reaction. The data in Table I show that the proteolytic coefficient is nearly constant for a range of enzyme concentration of at least 1 to 12. Since all of our assays were conducted within these limits, a true measure of the enzyme activity was obtained. The slightly decreased values of C^0 at the lowest enzyme concentrations indicate that some inactivation occurs during the long period necessary for conducting these assays. This is also indicated by the falling constants found in the experiment which contained 29 γ of protein N per cc.

Effect of Inhibitors and Metals—Table II shows that carnosinase is strongly inhibited by such typical metal poisons as sulfide, cyanide, and cysteine, and slightly inhibited by azide and fluoride. This suggests that the enzyme contains a metal which is essential for the activity. It also shows that most of the metal is retained by the protein during the purification procedure.

TABLE II

Effect of Enzyme Inhibitors on Carnosinase

The enzyme, Preparation A, was incubated 1 hour at 40° with the inhibitor and 0.01 M veronal buffer at pH 7.7 to 8.1 before addition of the substrate. No metal ions were added.

Inhibitor	Relative activity
	per cent
None	100
0.001 M sulfide	22
0.001 " cysteine	48
0.001 " azide	78
0.001 " fluoride	90
0.01 " "	76
0.001 " cyanide	95
0.01 " "	16
0.001 " iodoacetate	92

TABLE III

Effect of Various Metals on Activity of Carnosinase

The enzyme, Preparation A, was present at a concentration of 0.82 mg. of protein N per cc. in 0.01 M veronal buffer at pH 7.3 with the metal ions at 0.001 M.

Metal	Average, C^0	Relative activity
		per cent
Mn^{++}	0.92	172
Zn^{++}	0.73	136
None	0.56	100
Mg^{++}	0.49	87
Cd^{++}	0.44	79
Fe^{++}	0.42	75
Co^{++}	0.40	70
Ca^{++}	0.31	55

The metal-protein character of this enzyme is also indicated by the finding that Mn^{++} and Zn^{++} increase the activity (Table III). Other metals tested in this and additional experiments at other pH values produced either no effect or slight inhibition. The enhancement of activity produced by Mn^{++} in the experiment shown in Table III is actually minimal, since incubation of Mn^{++} and Preparation A for 3 hours at a somewhat higher pH value produced a greater effect. The data in Table IV demonstrate this time reaction between the protein and Mn^{++}. As with other peptidases (7), the time reaction indicates complex formation between the metal and the protein.

It is of considerable interest that the reaction kinetics are regular, even when insufficient incubation time is allowed for the maximal combination of the metal and protein in the absence of substrate. With other peptidases, increasing values of the velocity constants are obtained. With carnosinase we must assume that the substrate binds the metal with great avidity and prevents the development of the activity which would have been achieved in the absence of carnosine. Since the Mn^{++} concentration (0.001 M) is much lower than that of the substrate (0.05 M), there is ample substrate for binding most of the metal, and the actual concentration of free substrate is diminished to only a slight extent.

TABLE IV

Time Relations of Activation of Carnosinase by Manganous Ion

Preparation A (1.38 mg. of protein N per cc.) was incubated at 40° and at pH 7.75 with 0.01 M $MnCl_2$ in 0.03 M veronal buffer before addition to the flask containing the substrate. In the test solution, the Mn^{++} concentration was 0.001 M, the protein N, 0.55 mg. per cc., and the pH was 7.6.

Preincubation with Mn^{++}	Incubation with substrate	Hydrolysis	K^0	Average, C^0	Relative activation
hrs.	*hrs.*	*per cent*			*per cent*
0	2	19	0.16		
	4	41	0.17		
	4.5	43	0.16		
	6	50	0.14		
	6.75	62	0.15		
	8.25	74	0.15	0.27	
3	1	29	0.49		
	1.5	44	0.40		
	2	59	0.50		
	3	86	0.48	0.90	233
5	1.5	43	0.48	0.90	233

Although Zn^{++} activates carnosinase when present during the enzymatic assay, the data presented below indicate that the activation is extremely rapid under our experimental conditions.

Effect of pH and Buffer Ions—The data presented in Fig. 1 indicate that the optimal activity of the manganese-activated protein is in the region of pH 8.0 to 8.4, of the zinc-activated enzyme, at pH 7.8 to 7.9, and, without added metal, still lower at pH 7.4 to 7.5. It should be noted that the C^0 values plotted for the Mn^{++}-enzyme have been reduced to one-fourth of their true magnitude. Nevertheless, the significant shift in the pH optimum strongly suggests that the metal-enzyme in the tissues is not a manganese-protein. The optimum and the shape of the curve for the zinc-enzyme are closer to that of the unactivated preparation, and the smooth contours of both curves with the five different buffers indicate the absence of inhibition by these ions. The similarity of the unactivated enzyme and the zinc-enzyme suggests that natural carnosinase contains zinc.

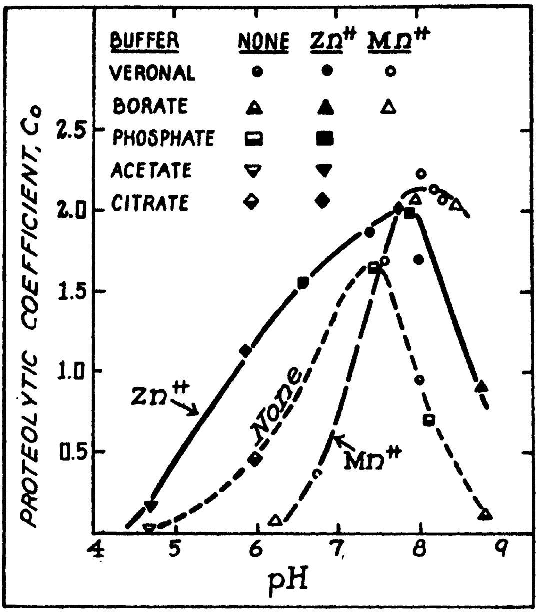

FIG. 1. Carnosinase activity as a function of pH in the presence of zinc, of manganese, and without added metal. Preparation A was exposed to Zn^{++} only during the course of the assays, but was preincubated with Mn^{++} for 3 hours. The C^0 values plotted for the Mn^{++} enzyme have been reduced to one-fourth of their actual magnitude. The enzyme concentration was 0.19 mg. of protein N per cc. The pH values were measured before 65 per cent of the substrate had been hydrolyzed.

The striking shifts in the pH activity curves indicate the necessity for careful evaluation of tests of metal activation. Had the effect of Mn^{++} been tested only at levels more acid than pH 6.7, the activation by this metal would have been missed completely. It is obviously desirable to test metal activation at several pH values.

The data of Table V demonstrate that the use of 0.08 M citrate in the reaction mixture reduces the activity of Preparation B to 18 per cent of its value in 0.05 M veronal at the same pH. Under the same conditions, phosphate ion reduces the rate to 10 per cent of that in veronal buffer. The data in Fig. 1 show that phosphate and citrate do not inhibit Preparation A (no added metal) or the zinc-enzyme preparation. Here again the difference between the unactivated and the manganese-enzymes indicates that manganese is not the original metal which must have been displaced by the relatively high concentration of manganese. The fact that the zinc-enzyme behaves like the unactivated enzyme is consistent with the view that zinc is the natural activator.

Phosphate or citrate buffers must be cautiously used in experiments with metal-enzymes, since these may have profound effects on these enzymes. With carnosinase, Zn^{++} appears to give greater activation than Mn^{++} in phosphate or citrate, whereas, with glycyl-L-leucine dipeptidase, these buffers produce an apparent activation because of their removal of Ca^{++} (8). Fortunately, the reaction rates of the Mn^{++}-enzyme in veronal and borate buffers are almost equal in the region of maximal activity (Fig. 1).

TABLE V

Inhibition of Manganese-Activated Carnosinase by Phosphate and Citrate

Preparation B was exposed to the buffers in the indicated concentrations only during the course of the hydrolysis. The final enzyme concentration was 0.18 mg. of protein N per cc.

Buffer	pH	C^{*}	Relative activity
			per cent
Veronal (0.05 M)	8.0	5.7	100
Phosphate (0.08 M)	8.1	0.44	8
" (0.08 ")	8.0	0.50	9
Citrate (0.08 M)	8.0	1.0	18

Stability of Carnosinase—The enzyme is much more stable in the presence of Mn^{++} than it is in that of Zn^{++} or in the absence of added metal. The instability of the Zn^{++}-enzyme was revealed by incubating the enzyme at 40° in 0.01 M veronal buffer. After 4 hours at pH 7.0, only 3 per cent of the activity remained, while, at pH 7.7, 63 per cent of the activity remained. This is in marked contrast to the 30 to 60 per cent increase in activity obtained when 0.001 M zinc acetate is present only during the hydrolysis of carnosine, even at pH 7.3. It is probable that the substrate protects the enzyme at these pH values. This phenomenon has been observed with other enzymes such as hexokinase (9).

It is notable that under the same conditions that obtain for the inactivation by zinc, manganese produces a 2- to 3-fold activation. The similar instability of the unactivated enzyme and of the Zn^{++}-enzyme at neutral pH values provides further evidence which points towards Zn^{++} as the metal in carnosinase in the tissues.

Differentiation of Carnosinase from Other Peptidases—Considerable evidence has been obtained which shows that the hydrolysis of carnosine by kidney extracts cannot be attributed to any known enzyme. For example, an aqueous extract of hog intestinal mucosa which contains all other known metal-activated peptidases has no hydrolytic action on carnosine; this has also been found to be true by Meshkova and Severin (10). A very active preparation of peptidases from human uterus (11) also was found to have no carnosinase activity. Crystalline bovine pancreatic carboxypeptidase at 0.5 mg. of protein N per cc., a concentration 1000 times greater than that required to split carbobenzoxyglycylphenylalanine, has no effect on carnosine or carbobenzoxycarnosine.

The inhibition of carnosinase activity by cysteine demonstrates that this enzyme is distinct from kidney cathepsins II, III, and IV, which are activated by cysteine (12). Moreover, kidney cathepsins I, II, III, and IV have optima in the range of pH 3.5 to 5.0, which is far more acid than the optimal pH for the hydrolysis of carnosine. The failure of iodoacetate to inhibit carnosinase also demonstrates that free sulfhydryl groups are unnecessary for the activity of the enzyme; this supplements the information gained with cysteine. Although the dehydropeptidase of kidney is activated by zinc (13), it differs from carnosinase in not being activatable by manganese. The known endopeptidases of the gastrointestinal tract do not contain metals. The present evidence appears to be sufficient to indicate that carnosinase is different from any of the known proteases.

Specificity of Carnosinase—In Table VI are presented the data for the action of Preparation B on a number of compounds containing histidine. It should be noted that the hydrolysis of certain of these compounds follows the kinetics of a first order reaction (C^1) more closely than those of a zero

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[1] It should be observed that no claim is made that Preparation B is enzymatically homogeneous. However, it is felt that the evidence is sufficient to indicate that the activity towards carnosine and the other histidine compounds represents a new type of peptidase activity. As already mentioned, crude extracts of hog intestinal mucosa and human uterus do not hydrolyze carnosine. This indicates that the carnosinase action cannot be attributed to such extremely active constituents of these and many other tissues as leucine aminopeptidase, glycylglycine dipeptidase, glycyl-L-leucine dipeptidase, prolidase, prolinase, aminotripeptidase, etc. However, Preparation B has been tested for some of these activities under the conditions of the experiments in Table VI. It was found that there was only a negligible amount of leucine aminopeptidase (3 per cent hydrolysis of L-leucinamide in 3.5 hours). Considerable hydrolysis of glycylglycine (54 per cent in 3.5 hours), of glycyl-L-leucine (69 per cent in 3.5 hours), and of triglycine (44 per cent of one peptide bond in 3 hours) was observed with this preparation.

order process. Both velocity constants have been given in order to facilitate comparison of the rates.

It has tentatively been assumed that the action on all of the histidine-containing substrates is due to the same enzyme. While further study is necessary to prove this assumption, certain definite conclusions may already be drawn from the available data. It is clear that no carboxypeptidase action or endopeptidase action is involved, since carbobenzoxycarnosine and carbobenzoxyglycyl-L-histidinamide are resistant to hydrolysis. It is

Table VI

Specificity of Carnosinase

The enzyme was Preparation B (0.19 mg. of protein N per cc.) in 0.04 M veronal buffer at pH 7.9 ± 0.1. The substrates were used at 0.05 M.

Substrate	Time	Hydrolysis	C^0	C^1
	hrs.	*per cent*		
β-Alanyl-L-histidine	0.5	61	5.3	
β-Alanyl-D-histidine*	24	20	0.074	
L-Alanyl-L-histidine*	0.25	23	8.0	0.040
	0.50	45	7.9	0.046
	0.75	61	7.2	0.048
	5	95		
D-Alanyl-L-histidine*	0.5	26	4.6	0.023
	0.75	41	4.8	0.027
	1.5	59	3.5	0.023
	24	98		
Glycyl-L-histidine*	0.5	35	6.1	0.033
	0.75	51	6.0	0.036
	1	64	5.6	0.039
	27	92		
L-α-Aminobutyryl-L-histidine*	2	15	0.68	0.0032
	3	19	0.58	0.0026
	22	87	0.37	0.0035
	49	99		
Glycyl-L-histidinamide	1	11	0.95	
	1.5	17	1.00	
	2	24	1.05	
	5	52	0.90	
	24	99		
β-L-Aspartyl-L-histidine*	48	18	0.006	
Carbobenzoxy-L-carnosine	48	0		
Carbobenzoxyglycyl-L-histidinamide	48	0		

* It is a pleasure to acknowledge the kindness of Dr. Vincent du Vigneaud in giving us these compounds and in providing us with seed crystals of carbobenzoxycarnosine and carnosine.

also apparent that the free amino group is essential for the enzymatic action.

If a single enzyme is involved, it would appear that the location and configuration of the amino group are not highly critical, since, in addition to the hydrolysis of carnosine, the preparation acts quite rapidly on glycyl-L-histidine, L-alanyl-L-histidine, and D-alanyl-L-histidine. The change in the position of the amino group from α to β or, at the α position, from the L to the D configuration affects the rate of hydrolysis only by a factor of 2. This is in great contrast to most enzymes in which substrates containing D-amino acids are highly resistant to proteolytic action. Moreover, with such enzymes as prolidase and glycyl-L-leucine dipeptidase, a shift in the amino group from the α to the β position decreases the sensitivity of the substrate about 300 times (1).

The lower rate of hydrolysis found with L-α-aminobutyryl-L-histidine indicates that the preparation acts most rapidly on compounds bearing a 2- or 3-carbon residue attached to the L-histidine moiety. The additional free carboxyl group of β-L-aspartyl-L-histidine markedly decreases the rate of hydrolysis of this peptide.

In contrast to the relative indifference of the preparation to the optical configuration of the alanine residue is the observation that D-carnosine (β-alanyl-D-histidine) is very resistant to hydrolysis. Obviously, the main specificity is directed towards the L-histidine portion of the substrate.

Glycyl-L-histidinamide is hydrolyzed and the action stops after one bond is split. This indicates that the hydrolysis occurs between the glycine and histidine residues. If the terminal amide linkage were split first, the resulting glycyl-L-histidine would be subject to the enzymatic action, and both bonds would be hydrolyzed. This interpretation of the locus of action on this substrate is supported by our failure to detect free ammonia in the test solution by direct nesslerization. Moreover, these results demonstrate that L-histidinamide is not hydrolyzed by Preparation B, and that there is no action of a histidine exoaminopeptidase. It was likewise found that carnosinase is not a β-alanine exoaminopeptidase, since no hydrolysis of β-alaninamide or β-alanylglycine could be observed.

It is clear from the present information that carnosinase is an exopeptidase like many other metal-peptidases. By the usual definitions the enzyme can be classified as a dipeptidase or an aminopeptidase. Although it is likely that the same enzyme acts on the various closely related histidine-containing peptides, we have used the name carnosinase in order to focus attention of the important naturally occurring substrate.

DISCUSSION

In an excellent summary of earlier work, du Vigneaud and Behrens (2) concluded in 1939 that "our knowledge concerning the origin, function, and fate of carnosine is still regrettably deficient." The results of the present investigation indicate the possible fate of this substance. The existence of a distinct enzyme in kidney and other tissues suggests that the normal metabolism of carnosine involves the hydrolysis of the peptide to its constituent amino acids which are then metabolized individually. Previous work (2) has already shown that injected carnosine causes an increased output of urea.

In a recent development of the polyaffinity theory, it was proposed (14) that the metal-peptidases combine with their substrates by the formation of a chelate complex in which the metal is the bridge between the protein and the substrate. It was also postulated that the bonds between the metal and the substrate are on opposite sides of the sensitive peptide linkage. With this notion applied to carnosinase, it would appear that one linkage of the metal is with the essential free amino group; the other would then have to be with the histidine-nitrogen of the peptide linkage. The striking shift in the pH activity curve of carnosinase with different metals provides additional evidence for this type of complex formation. A similar phenomenon has already been described for arginase by Hellerman and Stock (15).

EXPERIMENTAL

The enzymatic experiments were performed at a substrate concentration of 0.05 M. Hydrolysis was estimated on 0.2 cc. samples by titration of liberated carboxyl groups with 0.01 M potassium hydroxide in alcohol with thymolphthalein as the indicator (16). The results are expressed as 100 per cent for the complete hydrolysis of one peptide bond.

Carnosine

This was prepared essentially as described by Sifferd and du Vigneaud (6). However, several modifications were made which gave more convenient procedures, or more consistent yields. Carbobenzoxy-β-alanine methyl ester was converted to the hydrazide by shaking for 1 minute with a 2-fold excess of 85 per cent hydrazine hydrate in an equal volume of absolute ethanol; the product began to crystallize almost immediately and, after 3 hours at room temperature, the solution was cooled to 0°. A yield of 85 per cent was obtained.

Carbobenzoxycarnosine—More consistent yields of carbobenzoxycarnosine were obtained by allowing the reaction between carbobenzoxy-β-alanine azide with L-histidine methyl ester to proceed at 40° instead of at room temperature. After recrystallization from water, a preparation of carbobenzoxycarnosine was obtained which melted at 171°. Sifferd and du Vigneaud (6) reported 161°. To substantiate the higher melting point, the other properties were determined.

$C_{17}H_{20}O_5N_4$. Calculated. C 56.66, H 5.59, N 15.55
360.36 Found. " 56.65, " 5.60, " 15.53 (Dumas)

$$[\alpha]_D^{23} = +16.4° \text{ (1\% in water)}$$

Du Vigneaud and Hunt (17) found $[\alpha]_D^{24} = +10.5°$ (1 per cent in water). However, du Vigneaud and Behrens (2) cited a value obtained by Sifferd as $[\alpha]_D^{20} = +14.5°$.

Carnosine Nitrate—Hydrogenation of the above carbobenzoxycarnosine in the presence of 1 equivalent of nitric acid and a palladium catalyst gave a 92 per cent yield of the expected product; m.p. 223° (decomposition).

$$[\alpha]_D^{30} = +22.9° \text{ (5.7\% in water)}$$

Gulewitsch (18) reported $[\alpha]_D^{20} = +23.3°$ (5.2 per cent in water); m.p. 222° (decomposition).

Carbobenzoxyglycyl-L-histidinamide—Carbobenzoxyglycyl chloride (from 5.6 gm. of carbobenzoxyglycine) was coupled with L-histidine methyl ester (from 31.5 gm. of the dihydrochloride), as described by Hunt and du Vigneaud (19). The oily ester was allowed to stand for 2 days at room temperature in 100 cc. of methanol which had been previously saturated with NH_3 gas at 0°. The solution was concentrated *in vacuo*, and the residue extracted with 250 cc. of hot ethanol. The residual carbobenzoxy dipeptide amide was recrystallized from hot water. Yield, 1.9 gm.; m.p. 212–213° (decomposition). The compound apparently contained half a molecule of water which could not be removed at 93° *in vacuo*.

$C_{16}H_{19}O_4N_5 \cdot \frac{1}{2}H_2O$. Calculated. C 54.23, H 5.69, N 19.77
354.36 Found. " 54.36, " 5.78, " 19.87 (Dumas)

Glycyl-L-histidinamide Dihydrochloride—1.1 gm. of the above compound were hydrogenated in ethanol and water in the presence of 2 equivalents of HCl. The palladium catalyst was filtered, and the solution concentrated *in vacuo*. The product was recrystallized from ethanol ether; yield, 0.29 gm. The compound is exceedingly hygroscopic. For analysis, it was dried at 65° for 3 hours; it appears to be the monohydrate.

$C_8H_{13}O_2N_5 \cdot 2HCl \cdot H_2O$. Calculated. C 31.8, H 5.7, N 23.2
302.16 Found. " 31.8 " 6.0, " 22.8

SUMMARY

1. Liver, spleen, and kidney of the rat and swine kidney were found to be excellent sources of an enzyme, carnosinase, which hydrolyzes carnosine, β-alanyl-L-histidine.

2. The carnosinase of swine kidney has been partially purified. The enzyme is a metal-protein, as indicated by the inhibition produced by

metal-poisons such as cyanide and sulfide. Although both Mn^{++} and Zn^{++} activate the enzyme, it is likely that Zn^{++} is the metal which occurs in the enzyme in the tissue. This is indicated by the similarity of the Zn^{++}-enzyme and the unactivated preparation with respect to (*a*) pH of optimal activity, (*b*) the poor stability at 40° in the absence of substrate, and (*c*) insensitivity to phosphate and citrate.

3. Under the conditions of the experiments, the hydrolysis of carnosine follows the kinetics of a zero order reaction, and the rate is proportional to the enzyme concentration over a range of at least 1 to 12. The optimal action of the Mn^{++}-enzyme is at pH 8.0 to 8.4, of the Zn^{++}-enzyme at pH 7.8 to 7.9, and without added metal at pH 7.4 to 7.5.

4. Evidence has been given that the hydrolysis of carnosine cannot be attributed to any known protease. In addition to L-carnosine, the preparation hydrolyzes both L- and D-alanyl-L-histidine, glycyl-L-histidine, and glycyl-L-histidinamide. D-Carnosine, β-L-aspartyl-L-histidine, carbobenzoxy-L-carnosine, and carbobenzoxyglycyl-L-histidinamide are relatively resistant to hydrolysis. Carnosinase is an exopeptidase, and can be tentatively classified as a dipeptidase or an aminopeptidase.

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THE CHEMICAL NATURE AND MODE OF ACTION OF PANCREATIC CARBOXYPEPTIDASE*

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It has been shown that many of the exopeptidases are metal-protein compounds, and that the metal is essential for the hydrolytic action of these enzymes (1, 2). Although it is now some years since Anson (3) succeeded in obtaining the carboxypeptidase of bovine pancreas as a crystalline protein, no studies appear to have been made of the chemical characteristics of this enzyme, although some of the physical properties have been investigated by Putnam and Neurath (4). In the meantime, a wealth of data has accumulated on its specificity and kinetics (5–11).

Our first results (12) indicated that carboxypeptidase is a metallo-protein, since the enzymatic action is strongly inhibited by such typical metal poisons as cyanide and sulfide. In this investigation, a further study has been made of the action of various enzyme inhibitors which demonstrate more conclusively the essentiality and nature of the metal. In several instances, quantitative data have been obtained which indicate the number of moles of inhibitor which combine with each active group of the enzyme. This and other information has led to a picture of the enzyme-substrate complex and the method of action of the enzyme.

Unlike most of the other exopeptidases, the metal is firmly bound in carboxypeptidase. Ordinary precipitation procedures or dialysis do not remove the metal. Moreover, the inhibition which is produced by combination with metal-poisons is reversible by removal of the inhibiting agent. Magnesium has been tentatively identified as the metal in carboxypeptidase, since it was found by spectrographic analysis in the ash of the enzyme preparation (12). Quantitative analyses of digests of the crystalline enzyme are now in progress.[1]

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[1] In view of our somewhat unexpected finding that carboxypeptidase is a metal-protein, we have studied the action of trypsin in the presence of various inhibitors. Crystalline trypsin (the Armour Laboratories) was dialyzed until it was salt-free

EXPERIMENTAL

Crystalline carboxypeptidase was prepared from frozen bovine pancreas by the method of Anson (3) and recrystallized five times by the procedure of Neurath, Elkins, and Kaufman (8). The enzymatic experiments were performed in 2.5 cc. volumetric flasks at 25°. The extent of hydrolysis was measured on 0.2 cc. samples by the titration of liberated carboxyl groups (13).

We have used as the substrate in all of the experiments reported here carbobenzoxyglycyl-L-leucine (7) at a concentration of 0.05 M, although many preliminary experiments were also performed with carbobenzoxyglycyl-L-phenylalanine (12). Similar results were obtained with both substrates. It has already been shown that these substrates are hydrolyzed by the same enzyme (7).

The experiments were performed within the limits of pH 7.4 to 7.8; there seems to be little variation of the enzymatic activity within this pH region. Solutions of the inhibitors were adjusted within these pH limits by the addition of HCl or NaOH before adding them to the enzyme. Evaluation of the enzyme activity was made by calculation of the first order velocity constant K, where $Kt = \log (a/a-x)$ expressed in decimal logarithms, and of the proteolytic coefficient, C, which is K per mg. of protein N per cc. In many cases it was observed that falling velocity constants were obtained when enzyme, inhibitor, and substrate were added simultaneously. This could be avoided by allowing the enzyme and inhibitor to stand for a few hours at 25° or overnight at 2° before addition of the solution of the substrate. By this procedure we obtained reproducible first order velocity constants. Additional exposure of the enzyme to the inhibitor produced no further effect. Little or no inactivation of the enzyme could be detected in the control experiments, which were run parallel with the studies of inhibitor action.

In this study, several different preparations of carboxypeptidase were employed. These varied somewhat in their proteolytic coefficients and were of lower activity than some of the best preparations studied earlier (7, 8, 10). However, the extent of inhibition obtained with the different preparations was similar, and a few observations made with a carboxypeptidase of high specific activity ($C = 13$ for carbobenzoxyglycyl-L-phenylalanine) gave identical results. Presumably, our less active preparations contained some inert protein.

Kinetics in Presence of Inhibitor—Table I shows that in the presence of

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and then tested at pH 7.6 (phosphate buffer) for its action at 25° on α-benzoyl-L-argininamide. No inhibition could be detected in the presence of 0.01 M oxalate, 0.01 M citrate, 0.01 M fluoride, 0.001 M sulfide, or 0.001 M cyanide.

phosphate or of cyanide marked inhibition is obtained, as compared to the results with the control in veronal buffer. In each instance, the kinetics follow those of a first order reaction. No indication was obtained in these studies of the variation in first order constants recently described (11).

TABLE I

Hydrolysis of Carbobenzoxyglycyl-L-leucine by Carboxypeptidase in Presence of Inhibitors

The enzyme concentration was 0.004 mg. of protein N per cc. The cyanide solutions contained 0.04 M veronal buffer at pH 7.6. The phosphate experiments were performed with the sodium salts at pH 7.5 to 7.6.

	Time	Hydrolysis	$K \times 10^4$
	hrs.	*per cent*	
0.04 M veronal	0.5	22	36
	1.0	39	36
	1.5	51	34
	2.0	62	35
	2.5	71	36
	4.0	86	36
0.004 " phosphate	0.5	21	35
	1.0	36	32
	1.5	49	33
	2.0	59	32
	2.5	68	33
0.10 " "	1.0	15	15
	1.5	26	12
	2.5	36	13
	4.0	53	14
0.0015 M sodium cyanide	0.5	11	16
	1.0	18	14
	1.5	26	14
	2.5	43	16
	4.5	59	14
0.0005 " " "	0.5	15	24
	1.0	27	23
	1.5	38	23
	2.0	48	24
	2.5	54	22
	4.5	75	22

That inhibition is produced by high concentrations of phosphate must be emphasized in view of the many investigations on this enzyme in which this substance has been employed as a buffer (*e.g.* (11)).

The regularity of the kinetics indicates that a stable equilibrium has been reached between the enzyme-inhibitor complex and the free enzyme and inhibitor, as shown in equation (1).

$$\text{Enzyme} + \text{inhibitor} \rightleftharpoons \text{enzyme} - \text{inhibitor} \qquad (1)$$

Reversibility of Inhibition—That the reaction is indeed a reversible equilibrium has been shown by experiments in which removal of the inhibitor resulted in a restoration of the activity. The inhibition produced by phosphate appears to be instantaneously reversible on dilution. In the presence of 0.2 M phosphate C was 0.31, and after dilution to 0.002 M phosphate C was 1.27, whereas the original proteolytic coefficient of the preparation in veronal buffer was 1.38.

TABLE II

Reversibility of Carboxypeptidase Inhibition

The enzymatic tests were performed at 25° with 0.05 M carbobenzoxyglycyl-L-leucine as substrate at pH 7.5 ± 0.1 in the presence of a final concentration of veronal buffer at 0.04 M.

Preparation No.	Treatment	C^1
1	Control	1.30
2	With 0.003 M sodium cyanide	0.45
3	Preparation 2 after dialysis for 24 hrs. at 2° against 0.1 M veronal buffer	0.45
4	Preparation 3 after standing for 24 hrs. with 10 mg. horse methemoglobin at 25°	1.17
5	With 0.004 M sodium sulfide	0.60
6	Preparation 5 after dialysis for 24 hrs. at 2° against 0.1 M veronal buffer	0.82
7	Preparation 6 after standing for 24 hrs. with 10 mg. horse methemoglobin at 25°	1.30

Dialysis causes only a partial reversal of the sulfide inhibition (Table II), and no reversal of the cyanide inhibition. However, after dialysis, the activity is restored by the addition of small amounts of horse methemoglobin which has a high affinity for these inhibitors. Methemoglobin has no effect on control solutions of the enzyme. This technique of reversal of metal-enzyme inhibition has previously been used by Keilin and Hartree (14) to demonstrate the reversibility of the cyanide inhibition of uricase.

Other Inhibitors—The effect of various substances on carboxypeptidase is shown in Table III. The strong inhibition produced by citrate, oxalate, pyrophosphate, and cysteine all point to the presence of an essential metal in the enzyme. Moreover, the action of the first three inhibitors mentioned strongly suggests that magnesium is the responsible metal.

The negligible inhibition produced by fluoride, or by fluoride plus phosphate, is somewhat surprising, since fluoride has been regarded as a strong inhibitor of magnesium-enzymes. With enolase, Warburg and Christian (15) have shown that magnesium forms a reversible complex with fluoride and phosphate. Apparently with those enzymes with which the metal-protein combination is labile, a magnesium fluorophosphate is formed. In contrast to this, it appears that the magnesium is so tightly bound in carboxypeptidase that no fluoride or fluorophosphate compound can be formed. The strong binding of the metal in carboxypeptidase is demonstrated also by observations that, after the reversal of the inhibition produced by phosphate, cyanide, or sulfide, no activation resulted from the addition of magnesium, and that prolonged dialysis, first against 0.1 M phosphate and then against saline, does not remove magnesium, since almost the full activity of the enzyme is restored.

TABLE III

Action of Inhibitors on Carboxypeptidase

The substrate was carbobenzoxyglycyl-L-leucine (0.05 M). The solutions contained 0.04 M veronal buffer. The enzyme and inhibitor were incubated at 25° for 12 to 15 hours before the addition of the substrate. The inhibitor concentrations are for the final test solutions.

Inhibitor	pH	$K \times 10^4$	Inhibition
			per cent
Control in veronal buffer	7.4-7.9	40	
Sodium citrate (0.1 M)	7.6	9	78
" " (0.01 ")	7.5	32	19
" oxalate (0.1 M)	7.7	2.3	94
" " (0.01 ")	7.6	30	24
" fluoride (0.1 M)	7.8	41	0
" " (0.1 ") + phosphate (0.01 M)	7.5	39	0
" pyrophosphate (0.01 M)	7.8	0	100
" azide (0.1 M)	7.5	40	0
Cysteine (0.01 M)	8.0	0	100
Iodoacetate (0.01 M)	7.5	0	100
Silver nitrate (0.001 M)	7.7	40	0
Cuprous chloride (0.0001 M)	7.5	0	100
Mercuric " (0.0001 ")	7.5	40	0
Lead acetate (0.0001 M)	7.5	20	50

The inhibition by iodoacetate suggests the possible presence in the enzyme of sulfhydryl groups which are essential for the activity. This is also indicated by the inhibition produced by low concentrations of cuprous and lead ions. However, no inhibition was caused by the addition of silver and mercuric ions to the enzyme. Since these metals generally act as sulfhydryl inhibitors, the results cannot be easily interpreted at the present time. It may be tentatively assumed that the inhibition produced by iodoacetate, copper, and lead is due to reaction with sulfhydryl groups, and that the failure of the other heavy metals to inhibit is due to unknown factors.

The sensitivity of the enzyme to minute amounts of certain metals indicates the need for strict precautions in avoiding contamination by such substances during the preparation of carboxypeptidase. In a few instances, we have detected an apparent enzyme activation of 10 to 20 per cent in the presence of 10^{-4} M cyanide or sulfide. Ordinarily, this low concentration of these substances has no effect whatsoever. It is likely that these effects are due to the removal of traces of contaminating heavy metals.

The large amounts of sodium ion which were present in some of the inhibitor solutions led to experiments in which the activity of carboxypeptidase was tested with molar concentrations of sodium and potassium chlorides. These gave enzymatic activities which were identical with the control experiments in veronal buffer of lower ionic strength.

Quantitative Inhibition Studies—Studies were made with several inhibitors in order to determine the quantitative relationship of inhibitor to the active groups of the enzyme. For an equilibrium of the kind shown in equation (1), where a is the total enzyme concentration, x is the amount of active enzyme, and $(a - x)$ is the quantity of enzyme bound to the inhibitor, then

$$KI^N = \frac{(a - x)}{x} \tag{2}$$

where I is the concentration of inhibitor, N is the number of moles of inhibitor combined with one active group of the enzyme, and K is the equilibrium constant.

Equation (2) yields curves of different shape where N has different values; these are shown in Fig. 1, where N is 1, 2, or 4. On the semilogarithmic plot, the shape of the curves is independent of the parameters where the maximal enzyme activity is plotted to the same scale.

Fig. 2 shows the results obtained with phosphate. The curve drawn through the data is the theoretical one from equation (2) where N is equal to 1. This suggests that 1 mole of phosphate combines with one active center of the enzyme. The curve where N is equal to 2 does not give a satisfactory description of the data.

Fig. 3 presents the results for sulfide inhibition. Like the phosphate data, the curve which best describes the results is the one from equation (2) where N is equal to 1. Curves with other values of N do not describe the results. With the exception of the value obtained at the highest sulfide concentration, the fit is quite good. At the high concentrations of inhibitor, precise data are difficult to obtain, since the time required for kinetic measurements is quite long, and during this time some irreversible inactivation of the enzyme may occur.

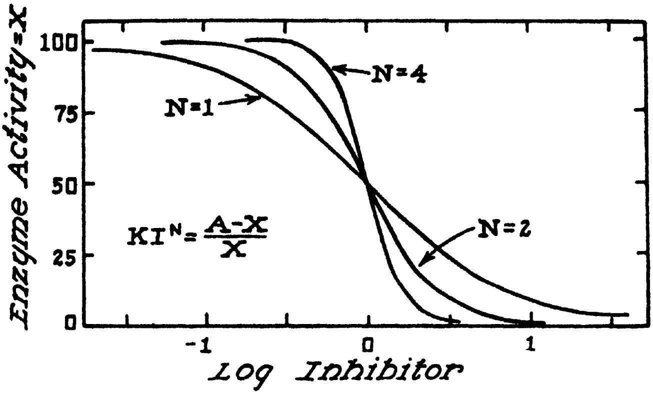


FIG. 1. Theoretical curves obtained from equation (2), where N has the value of 1, 2, or 4.

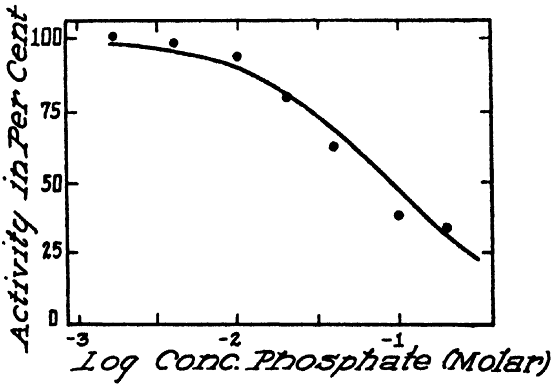


FIG. 2. Effect of orthophosphate concentration on activity of carboxypeptidase. The curve drawn through the data is from equation (2), where N is 1. In these experiments and those given in Figs. 2 and 3, a constant amount of enzyme was incubated with the inhibitor for several hours at 25° and at pH 7.5 ± 0.1. The inhibitor concentration is given for the final test solution after addition of the substrate (0.05 M carbobenzoxyglycyl-L-leucine). The final enzyme concentration was 0.004 mg. of protein N per cc. No buffer other than phosphate was present in these experiments. The inhibitor concentration is the total amount of orthophosphate present.

The data shown in Fig. 4 are for the effect of cyanide on carboxypeptidase activity. Here the best description of the results is obtained with the curve from equation (2), where N is equal to 2. Like the results obtained with cyanide and sulfide, the extensive data obtained with this inhibitor are sufficiently precise to eliminate easily other values of N from consideration. This result leads to the conclusion that 2 moles of cyanide combine with each active center of the enzyme, while the binding of only one phosphate or one sulfide by the active group produces inhibition.

The concentration of these substances required for 50 per cent inhibition (equivalent to K) is 0.089 M phosphate, 0.0037 M sulfide, and 0.00107 M cyanide.

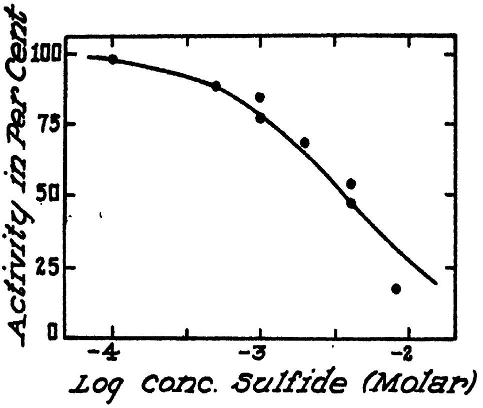


FIG. 3

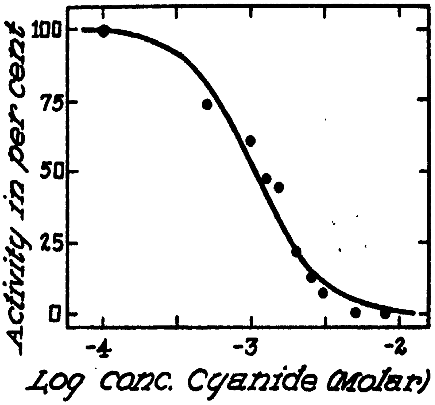


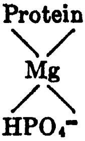
FIG. 4

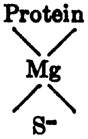
FIG. 3. Effect of sodium sulfide concentration on activity of carboxypeptidase. The smooth curve is from equation (2), where N is 1. Veronal (0.05 M) was used as the buffer. The other conditions of the experiments were the same as those given in Fig. 2.

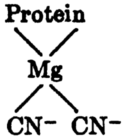
FIG. 4. Carboxypeptidase activity as a function of sodium cyanide concentration. The curve is derived from equation (2), where N is 2. Veronal (0.05 M) was used as the buffer. The experiments were performed in the same manner as those presented in Fig. 2.

DISCUSSION

The inhibition data obtained with carboxypeptidase indicate that this enzyme is a metallo-protein in which the metal is essential for the activity. It has already been suggested that in the metal-peptidases the rôle of the metal is to form a chelate complex with both the protein and the substrate (1). With glycylglycine dipeptidase, there is marked parallelism between the ability of cobaltous ion to form a chelate complex with the substrate, as observed spectroscopically, and the ability of the enzyme to hydrolyze the substrate. With carboxypeptidase, the quantitative inhibition studies suggest that two bonds are available for formation of the inhibitor complex or of the enzyme-substrate complex.





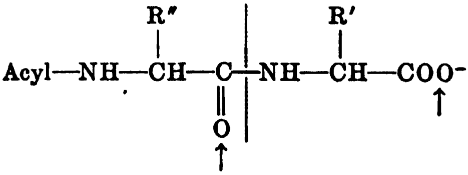


Thus with this metal which can form four bonds, two of coordinate and two of covalent character, there are two valences which can combine with the inhibitor, and presumably two which are strongly linked with the protein.

While the schematic diagrams indicate the correct ratios of combination, it is not implied that each magnesium combines with both valences in $\mathrm{S}^{=}$ or $\mathrm{HPO}_4^{=}$. It is more likely that these ions actually form bridges which link the active groups of two molecules, as in —Mg—S—Mg—S—, or $-\mathrm{Mg}-\mathrm{HPO}_4-\mathrm{Mg}-\mathrm{HPO}_4-$. However, the ratios indicate the important result that two valences of the metal are available for combination with the inhibitor.

Carboxypeptidase apparently contains sulfhydryl groups, as suggested by the strong inhibition of the enzyme which is produced by iodoacetate and by low concentrations of Cu^{+} and Pb^{++}. It is reasonable to assume that, in the absence of substrate or inhibitor, two valences of the metal are tightly bound to unknown groups of the protein and two are loosely bound to sulfhydryl groups. In the presence of inhibitors or of a suitable substrate, the loose combinations are displaced by those compounds for which the metal has a higher affinity.

The typical substrates of carboxypeptidase do not contain a free amino group adjacent to the sensitive bond but must have a free carboxyl group (5, 10). A peptide hydrogen is not essential in the substrate, as indicated by the slow enzymatic hydrolysis of carbobenzoxy-L-tryptophyl-L-proline (10) and, even more dramatically, by the rapid hydrolysis of an ester bond in the compound hippurylphenyllactic acid (9). Chelate complexing of the metal must then occur with the carbonyl group at the sensitive bond and with the ionized carboxyl group, as shown for an acylated peptide.



$$\mathrm{Acyl{-}NH{-}\overset{\overset{\displaystyle R''}{|}}{C}H{-}\underset{\underset{\displaystyle \uparrow}{\overset{\|}{O}}}{C}\;\vdots\;NH{-}\overset{\overset{\displaystyle R'}{|}}{C}H{-}\underset{\uparrow}{COO^{-}}}$$

The arrows show the points of metal combination and the dotted line the sensitive bond. In contrast with dipeptidases and aminopeptidases where chelation of the metal must occur with the free amino group and the nitrogen of the sensitive peptide linkage, the metal in carboxypeptidase does not combine with nitrogen at all. It should be noted that the substrate combines not only with the metal but also directly with the protein through the center that binds R′; the nature of R′ determines in large part the specificity of action and the rate of hydrolysis of different substrates (7, 10). It is also likely that R″ may be bound by the protein, but the effect is small as compared to the large effect of R′ (10, 16).

The rôle of the metal in forming the enzyme-substrate complex of the peptidases has led to the development of a theory of the mode of action of these enzymes (16). Briefly, it is postulated that, in each instance, chelation occurs at opposite sides of the sensitive peptide bond. These two linkages (together with those which involve the protein directly through R′ and R″) produce a strain or electronic distortion at the sensitive linkage; this labilization (decrease in the energy of activation) permits the hydrolysis of the linkage by the usual hydrogen or hydroxyl ion catalysis. While this hypothesis is now being subjected to further experimental test, it has already led to an explanation of many hitherto unrelated facts concerning carboxypeptidase (16).

It is noteworthy that according to this hypothesis the strength of the acid combined at the sensitive linkage has a tremendous effect on the rate of hydrolysis of the peptide bond. For example, it is well known that chloroacetylamino acids are much more sensitive to hydrolysis than acetylamino acids; the former are much stronger acids than the latter. Similarly, it was demonstrated (17) that carbobenzoxy-β-alanyl amino acids are hydrolyzed much more slowly than carbobenzoxyglycyl amino acids. It has now been found that carbobenzoxyglycine is a much stronger acid than carbobenzoxy-β-alanine.[2] Obviously, this is a free energy effect similar to those reported for the non-enzymatic hydrolysis of many homologous series of esters and amides (18).

The specificity of carboxypeptidase (as well as other metal-peptidases) may now be analyzed in terms of a number of distinct factors: (1) the diaffinity concept of von Euler and Josephson (19), which may be interpreted in terms of the necessary polar groups of the substrate which are bound by the metal; (2) the polyaffinity concept of Bergmann (20), which is concerned with the correct steric relationship of R′ and the essential polar groups; (3) the specific binding energy of the protein for R′, R″, and possibly other side chains, depending on the nature of the enzyme and its substrates; and (4) the free energy of the sensitive linkage which depends on the acid strength of the carboxyl group and on the strength of the amino or other group which is linked to the acid. There are undoubtedly many additional factors which influence the specificity of peptidases, and it will now be possible to separate them from these known factors which influence the specificity of these enzymes.

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[2] Unpublished observations by Lumry, Polglase, and Smith.

SUMMARY

1. Crystalline pancreatic carboxypeptidase is inhibited by cyanide, sulfide, phosphate, pyrophosphate, citrate, oxalate, and cysteine. These studies indicate that a metal, presumably magnesium, is an essential constituent of the enzyme.

2. Each active group of the enzyme combines reversibly with 1 mole of phosphate, 1 mole of sulfide, and 2 moles of cyanide.

3. Carboxypeptidase is inhibited by iodoacetate and by Cu^+ and Pb^{++} ions, suggesting the presence of essential sulfhydryl groups.

4. This information has led to a picture of the method of combination of enzyme and substrate, and a theory of the mode of action of the metal-peptidases.

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PAPAIN RESOLUTION OF DL-TRYPTOPHAN; OPTICAL SPECIFICITY OF CARBOXYPEPTIDASE*

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The optical specificity of the synthetic activity of papain has previously been used for the resolution of the DL mixtures of derivatives of several amino acids (1–3). With cysteine-activated papain and a mixture of the carbobenzoxy-DL-amino acid and aniline, the carbobenzoxy-L-amino acid anilide is synthesized and crystallizes from the solution. The carbobenzoxy-D-amino acid is readily recovered from the mother liquor.

We have used this method to resolve carbobenzoxy-DL-tryptophan. The crystalline carbobenzoxy-L-tryptophan anilide was obtained in quantitative yield, but no method was found by which the free L-tryptophan could be prepared without extensive destruction or racemization.[1] However, the carbobenzoxy-D-tryptophan was easily converted by hydrogenation to D-tryptophan. Peptide derivatives of D-tryptophan were prepared.

Previous studies (4) have demonstrated that substrates which possess the free carboxyl group on a terminal D-amino acid are extremely resistant to the hydrolytic action of crystalline pancreatic carboxypeptidase. In agreement with these observations, we find that carbobenzoxyglycyl-D-tryptophan is not hydrolyzed by this enzyme (Table I). However, substrates of the type of carbobenzoxy-D-tryptophylglycine have not yet been tested. We find that this compound is also resistant to the hydrolytic action of carboxypeptidase (Table I). Thus, it appears that, for the action of this enzyme on carbobenzoxy dipeptides, the amino acid residue at either position cannot be of the D configuration.

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[1] It is apparent that hydrolysis by strong acid or alkali is not suitable for the preparation of this amino acid. L-Tryptophan may be recovered by hydrogenation of carbobenzoxy-L-tryptophan anilide followed by the papain hydrolysis of L-tryptophan anilide. However, the objective in this investigation was a simple method for the preparation of D-tryptophan.

EXPERIMENTAL

The crystalline carboxypeptidase was obtained from frozen bovine pancreas by Anson's method (5) and recrystallized five times by the procedure of Neurath, Elkins, and Kaufman (6). The enzymatic tests were performed in 2.5 cc. volumetric flasks. Hydrolysis was measured on 0.2 cc. samples by the carboxyl titration method of Grassmann and Heyde (7).

The analyses for nitrogen on the compounds described below were performed by the micro-Dumas method.

*Enzymatic Synthesis of Carbobenzoxy-*L*-tryptophan Anilide*—61 gm. of carbobenzoxy-DL-tryptophan (8) were pulverized and dissolved in 90 cc. of 2 M NaOH, and there were added 28 cc. of reagent grade aniline, 650 cc. of 0.2 M citrate buffer at pH 5.0, 1.5 gm. of cysteine hydrochloride in a few cc. of water, and a filtered solution of the enzyme obtained by extracting 4 gm. of commercial papain[2] with 30 cc. of water. The mixture was diluted with water to about 1 liter and allowed to stand at 40° for 6 days. In this reaction mixture, adjusted near the pH optimum of papain, carbobenzoxy-DL-tryptophan is only partially soluble. Therefore, at 2 day intervals, the incubation was interrupted by cooling[3] the preparation to 0° and adding 2 M NaOH until the solution was alkaline to phenolphthalein; the insoluble anilide was then filtered. After each of the first two crops was removed, the filtrate was readjusted to pH 5 with 5 M HCl, and enzyme and cysteine were added as before. The three crops of anilide (needles) weighed 37 gm. equivalent to 100 per cent of the theoretical yield. After

TABLE I

Action of Crystalline Carboxypeptidase on Peptide Derivatives of L*-Tryptophan and* D*-Tryptophan*

The tests were performed at 25° with solutions of 0.05 M substrate at pH 7.6 buffered with 0.04 M veronal.

Substrate	Protein N per cc. test solution	Time	Hydrolysis
	mg.	*hrs.*	*per cent*
Carbobenzoxyglycyl-L-tryptophan	0.0005	2.5	58
Carbobenzoxyglycyl-D-tryptophan	0.4	54	0
Carbobenzoxy-L-tryptophylglycine	0.4	4	57
Carbobenzoxy-D-tryptophylglycine	0.4	54	0

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[2] Wallerstein Company, Inc., New York.

[3] Alkali must be added only to cold solutions; otherwise extensive racemization occurs. In a trial experiment where this precaution was not taken, about 75 per cent of the carbobenzoxy-DL-tryptophan which was used was recovered as carbobenzoxy-L-tryptophan anilide.

recrystallization from ethyl acetate-petroleum ether, the compound melted at 172–173°.

$C_{25}H_{23}O_3N_3$. Calculated. C 72.6, H 5.6, N 10.2
413.5 Found. " 72.4, " 5.9, " 9.8
$[\alpha]_D^{28} = +39.2°$ (2.5% in glacial acetic acid)

This anilide was also prepared by coupling carbobenzoxy-L-tryptophyl chloride (8) with aniline. After recrystallization from ethyl acetate-petroleum ether,

Calculated, N 10.2; found, 9.9
$[\alpha]_D^{28} = +39.4°$ (2.5% in glacial acetic acid)

Carbobenzoxy-D-tryptophan—After the removal of the last crop of carbobenzoxy-L-tryptophan anilide, the filtrate was acidified to Congo red by the dropwise addition of 5 M HCl, and allowed to stand at 0° for 24 hours. The yield was 27 gm. or 87 per cent of the theoretical. The compound was dissolved in ethyl acetate, treated with norit, and recrystallized by the addition of petroleum ether; m.p., 124–126°.

$C_{19}H_{18}O_4N_2$ (338.4). Calculated, N 8.3; found, N 7.9
$[\alpha]_D^{22} = +15.4°$ (5% in 1 equivalent of M NaOH)

Carbobenzoxy-L-tryptophan[4] melts at 126° (8), and has an $[\alpha]_D^{22} = -14.8°$ (5 per cent in 1 equivalent of M NaOH).

D-*Tryptophan*—4.0 gm. of carbobenzoxy-D-tryptophan were hydrogenated in 40 cc. of methanol, 3 cc. of water, and 1 cc. of glacial acetic acid in the presence of palladium black. About 250 cc. of hot water were added to dissolve the product before filtering the catalyst. The hot aqueous solution was treated with norit and concentrated *in vacuo*. Yield, 1.8 gm.

Berg (9) found for D-tryptophan, $[\alpha]_D^{25} = +32.45°$ (0.5 per cent in water); Dunn and Rockland (10) give for L-tryptophan, $[\alpha]_D^{25} = -32.15°$ (2.07 per cent in water).

$C_{11}H_{12}O_2N_2$ (204.1). Calculated, N 13.7; found, N 13.6
$[\alpha]_D^{26} = +33.1°$ (0.8% in water)

Carbobenzoxy-D-tryptophylglycine—2 gm. of carbobenzoxy-D-tryptophan were converted to the crystalline acid chloride (m.p. 75°) as previously described for the corresponding L compound (8). A solution of the acid chloride in 20 cc. of ethyl acetate was coupled at 0° with 0.53 gm. of glycine in the usual manner by using M NaOH as the neutralizing agent. After 30 minutes at room temperature, the solution was acidified to Congo red with 5 M HCl, and the product was extracted into ethyl acetate. The solution was dried over Na_2SO_4, and the product crystallized by addition of petroleum ether. Yield, 1.5 gm. After recrystallization from the same solvents, the melting point was 156.5°.

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[4] The optical activity of these L compounds was measured on the identical preparations described earlier (8).

$C_{21}H_{21}O_5N_3$ (395.4). Calculated, N 10.6; found, N 10.3
$[\alpha]_D^{26}$ = +20.2° (4.7% in absolute ethanol)

For the corresponding L compound, melting point 156° (8), and $[\alpha]_D^{26}$ = −19.8° (4.7 per cent in absolute ethanol).

Carbobenzoxyglycyl-D-tryptophan—To a solution at 0° of 2.5 gm. of D-tryptophan in 12 cc. of M NaOH, 3.1 gm. of crystalline carbobenzoxyglycyl chloride were added with an additional 12 cc. of M NaOH. After standing in the cold for 20 minutes and at room temperature for 1 hour, the solution was acidified and the product extracted into ethyl acetate. The solution was washed with water, and concentrated *in vacuo*. The oily residue was dissolved in hot ethanol-water and treated with norit. The crystalline product (2.6 gm.) melted at 70°; this was assumed to be an alcoholate. After recrystallization from ethyl acetate-petroleum ether, the melting point was 141°.

$C_{21}H_{21}O_5N_3$ (395.4). Calculated, N 10.6; found, N 10.3
$[\alpha]_D^{22}$ = −33.4° (2.5% in absolute ethanol)

The corresponding L compound[4] melts at 142° (8), and has $[\alpha]_D^{22}$ = +33.5° (2.5 per cent in ethanol).

SUMMARY

1. Carbobenzoxy-DL-tryptophan has been resolved by the action of cysteine-papain in the presence of aniline. The anilide of the L isomer separated in crystalline form. From the filtrate carbobenzoxy-D-tryptophan was obtained which was then converted to D-tryptophan. Peptide derivatives of D-tryptophan were prepared.

2. Carboxypeptidase has no hydrolytic action on carbobenzoxyglycyl-D-tryptophan or carbobenzoxy-D-tryptophylglycine.

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THE EFFECT OF THYROID ACTIVITY ON CERTAIN ANABOLIC PROCESSES STUDIED WITH THE AID OF DEUTERIUM*

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In the study of the metabolic defects of hypo- and hyperthyroidism, great stress has been placed upon the alterations of the total metabolic rate, as measured by gaseous exchange, which in turn represents the summation of many simultaneous processes, among which the catabolic ones predominate. The isotope technique has provided a convenient method for the estimation of the rates at which certain tissue constituents are being synthesized and deposited in the intact animal body. By replacement of a portion of the body water by deuterium oxide and observation of the rates at which stably bound deuterium appeared in tissue fatty acids (1), cholesterol (2), and glycogen (3), it has been possible to estimate the rates of synthesis of these several tissue constituents under various circumstances. It was considered of interest to apply this technique to the study of the anabolic rates of various tissue constituents of experimental animals at different levels of thyroid activity.

To this end, a group of rats was rendered thyrotoxic by the addition to their diet of desiccated thyroid gland.[1] Hypothyroidism was produced in a second series of rats by the administration of thiouracil (4);[2] and a third group of rats, receiving no medication, served as controls. Immature rats were selected as experimental animals in view of their favorable and relatively rapid response to thiouracil (4).

EXPERIMENTAL

Animals and Diet—Immature female rats of the Sherman strain were used throughout. At the age of 22 days, rats were placed in metabolism

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[1] Thyroid, U. S. P., Parke, Davis and Company.

[2] The authors wish to express their gratitude to Lederle Laboratories Division, American Cyanamid Company, for their gift of Deracil powder.

cages, two rats per cage, and divided into three groups, litter mate controls being used as far as possible. The basal diet comprised 60 parts of corn starch, 22 parts of casein, and 6 parts each of yeast powder, roughage (Celluration), and salt mixture (5). Initially four rats were employed for the determination of each experimental point in each of the three groups, but as the mortality rate among the animals receiving thyroid substance was high, supplementary rats were added to furnish sufficient material. A total of 82 rats was employed in the entire experiment. Food and water were allowed *ad libitum* and the quantity consumed was estimated daily. Weekly supplements of vitamin A and D concentrate were administered to all rats, and additional small supplements of cod liver oil and linseed oil were given from time to time to those rats receiving thyroid gland.

The first group of rats served as normal controls. To the drinking water of the animals of the second group was added 0.1 per cent of thiouracil. The thiouracil consumed increased from an initial value of about 5 mg. to about 10 mg. per rat per day at the end of the experiment. With the diet of the third group was admixed 1 per cent of desiccated thyroid powder. Except for retarded growth (*cf.* Fig. 1), no untoward symptoms were noted among the animals receiving thiouracil. After about 2 weeks the rats fed thyroid gland exhibited all the expected signs of toxicity: hyperactivity, tremor, tachycardia, and cachexia. The mortality in this group was 47 per cent and the animals which were subjected to autopsy showed marked congestion of all internal organs. The weight curve of this group is distinctly subnormal (*cf.* Fig. 1). The average daily food consumption of the three groups during the last week of life was 12 gm. for the normal rats, 12 gm. for the rats receiving thiouracil, and 22 gm. per 100 gm. of body weight per day for the rats receiving thyroid substance.

All thyroid glands were studied grossly and histologically. The glands from those animals which had received thiouracil were grossly enlarged, hyperemic, firm, and readily separable from the tracheas. Histologically, the epithelium lining the follicles was hyperplastic and hypertrophic, and there was great loss of colloid and invagination of the lumen of the follicles. The thyroid glands from the rats which had been fed thyroid substance were much smaller than normal, pale, and not firm. In many cases they were distinguishable with difficulty from surrounding tissues, even under the dissecting microscope. Histologically, the epithelium lining the follicles was flattened and, in many areas, atrophied; the follicles were small and filled with dense colloid. No difficulty was encountered in classifying the microscope slides according to the treatment which the animals had received during life. The histological results were confirmed by Dr. H. C. Stoerck and Dr. D. G. Worcester of the Department of Pathology, College of Physicians and Surgeons.

Deuterium Oxide Administration—Subgroups of at least four animals each, from each of the three groups, were given D_2O for periods of ½, 1, 2, 4, and 8 days. The initiation of the isotope regimen was so adjusted that all experiments were terminated on the 45th day of life. At the appropriate time, each rat received a subcutaneous injection of 1 ml. per 100 gm. of body weight of 99.5 per cent D_2O containing 0.9 per cent NaCl. From this time on, drinking water was replaced by 1.7 per cent D_2O, and this solution was also used to moisten the food. This procedure served to establish and maintain D_2O concentrations of 1.1 to 1.2 per cent in the body water of the animals.

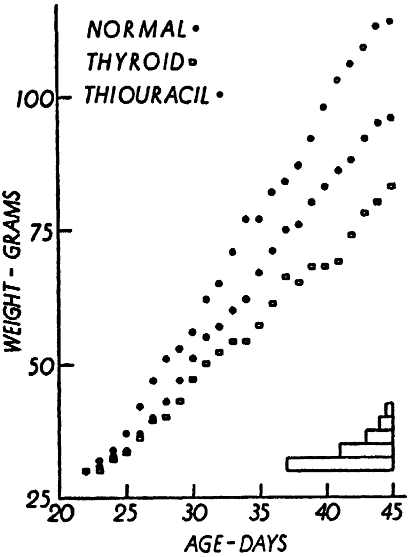

FIG. 1. Growth curve of animals. The average weights of the total number of animals within each group are plotted against age throughout the period of medication. The hollow blocks (right corner) represent the duration of the isotope regimens.

At exactly 45 days of age, each rat was killed by the intraperitoneal injection of nembutal.

Isolations and Analyses—The tissues from the animals of each subgroup were pooled for analysis. Livers were rapidly removed, weighed, and ground with sand in a volume of cold 10 per cent trichloroacetic acid approximating the weight of the tissue. This mixture was filtered by suction and the solid residue extracted twice again with cold 5 per cent trichloroacetic acid. From the combined filtrates glycogen was immediately precipitated by the addition of ethanol to a final concentration of 60 per cent (6). Purification of the glycogen was effected by successive reprecipitations with ethanol, once from 15 per cent KOH solution, and twice from water. The white product was washed by centrifugation with increasing concentrations of ethanol and then acetone, was air-dried, and finally dried *in vacuo* over P_2O_5.

The residue from the trichloroacetic acid extractions was further extracted by boiling successively with 95 per cent ethanol, ethanol-ether mixture (3:1), and twice with ether. The ether was evaporated from the combined lipide extract and the remaining ethanolic solution made alkaline by the addition of 5 per cent of solid KOH. The solution was boiled under a reflux for 3 to 4 hours, diluted with an equal volume of water, and the non-saponifiable portion exhaustively extracted with petroleum ether. From a solution of the non-saponifiable fraction in 80 per cent ethanol, sterol digitonides were precipitated and isolated.

The aqueous-ethanolic solution of saponifiable products was acidified to Congo red paper, and the liberated fatty acids were extracted with petroleum ether. The combined petroleum ether solution of fatty acids was washed with water, dried over Na_2SO_4, evaporated to dryness, and the residue dried *in vacuo* over P_2O_5 and paraffin.

The residual liver tissue which remained after the foregoing extractions was dried overnight at 90°, ground thoroughly with the sand with which it was contaminated, and aliquot portions taken for micro-Kjeldahl nitrogen determinations. This insoluble fraction has been designated "liver protein," and its quantity estimated from the nitrogen determination.

The eviscerated carcasses of the rats from each subgroup were ground and digested in hot aqueous 30 per cent KOH, the volume of the reagent approximating the mass of tissue. The alkaline digest was filtered through glass wool and glycogen was precipitated from the filtrate by the addition of 1.2 volumes of ethanol. The glycogen was collected by sedimentation and centrifugation and was purified by successive precipitations with ethanol, once from 10 per cent aqueous trichloroacetic acid, and twice from water.

The isolations of carcass sterols and carcass fatty acids were conducted on a weighed aliquot portion of the aqueous-ethanolic alkaline supernatant solution from which glycogen had been precipitated. The steps were similar to those described above in the treatment of liver.

The pooled hearts, lungs, spleens, and kidneys from each subgroup were used for the isolation of a sample of body water (7). The segment of trachea to which the thyroid gland adhered was removed from each animal, and the thyroid gland was subsequently freed of adjacent tissues under the dissecting microscope. The gland was fixed in Bouin's solution, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin. The elapsed time between the killing of the animal and the disposition of the carcass was 5 to 6 minutes. All deuterium analyses were carried out by the falling drop technique (8).

DISCUSSION

That the animals receiving thyroid substance exhibited the expected toxic response was shown by the development of the symptoms of thyrotoxicosis, the high mortality, and the inhibition of gain in weight despite polyphagia (9). Whereas the normal rats, during the last 12 days of the experiment, gained an average of 0.33 gm. in weight per gm. of food eaten, the rats receiving thyroid gland gained only 0.14 gm. The complete absence of glycogen from the livers of the animals receiving thyroid gland was a uniform observation in the present series and is in accord with numerous reports of this effect of thyroid intoxication (10). Relative, as well as absolute, hepatomegaly was noted (Table I); liver weight per 100 gm. of body weight averaged 4.53 gm. for the normal animals, and 7.23 gm. for the rats receiving thyroid gland, a finding which has previously been described (11). The grossly evident cachexia was reflected in the analytically determined diminution in the quantity of depot fat; the rats receiving thyroid gland contained almost 25 per cent less depot fat per 100 gm. of body weight than did the normal control series. The histological changes observed in the thyroid glands of these animals were in accord with the expected response to thyroid administration (12, 13).

Table I

Carcass and Liver Weights; Deuterium Content of Body Water

Duration	Normal				Thiouracil				Thyroid			
	No. of animals	Carcass weight per rat	Liver weight per rat	Body H_2O	No. of animals	Carcass weight per rat	Liver weight per rat	Body H_2O	No. of animals	Carcass weight per rat	Liver weight per rat	Body H_2O
days		*gm.*	*gm.*	*atoms per cent excess D*		*gm.*	*gm.*	*atoms per cent excess D*		*gm.*	*gm.*	*atoms per cent excess D*
½	4	108	4.88	1.16	4	105	4.86	1.22	4	88	7.03	1.22
1	4	127	5.46	1.17	4	109	5.61	1.19	2	79	5.28	1.21
2	4	111	5.24	1.21	4	80	3.92	1.17	3	81	5.23	1.11
4	4	120	5.06	1.09	8	90	4.13	1.16	6	80	5.55	1.16
8	4	106	5.19	1.18	6	100	4.40	1.18	4	86	6.57	1.14
Weighted average.......		114	5.16			96	4.50			83	6.00	

The evidence that thiouracil had exerted its inhibitory effect in our animals rests largely upon the gross and histological appearance of the thyroid gland itself, which was identical with the descriptions in the literature (14, 15). The correlation between this appearance of the gland and lowered metabolic rate has been demonstrated by others (16, 17). The moderate retardation of growth in response to thiouracil (*cf.* Fig. 1) was also in accord with the reports of others (14, 15). No significant deviation from normal food intake was noted.

Glycogen—No trace of glycogen was recovered from the livers of any of the rats receiving thyroid gland. The deuterium concentrations in the successive samples of liver glycogen obtained from the normal and thiouracil-treated rats varied widely (*cf.* Fig. 2), a finding which has been previously observed (3).

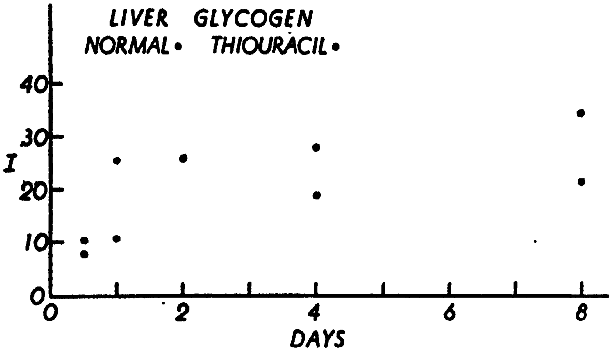

FIG. 2. Deuterium concentrations in liver glycogen samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

Comparison of the deuterium concentrations in the samples of carcass glycogen from the normal and the thiouracil-treated rats (Fig. 3) reveals no striking differences. On the other hand, point for point, the samples of carcass glycogen from the rats receiving thyroid gland were richer in deuterium than those from the other two groups. The three factors which determine the rate of increase in deuterium of a tissue constituent in such an experiment are the amount synthesized each day, the amount of preexisting non-isotopic tissue constituent with which this is diluted, and the actual isotope concentration in the newly synthesized product. This last quantity may be evaluated experimentally by estimation of the asymptotic value which the isotope concentrations appear to be approaching. From Fig. 3 it would appear that this asymptote lies in the neighborhood of 35 to 40 atoms per cent D for the glycogen in the thyroid-treated animals, and at 15 to 20 atoms per cent D for the glycogen in the other two groups. This suggests that when thyroid substance was fed, approximately twice as much stably bound hydrogen in the newly synthesized glycogen had arisen from the body water as was the case in the other two groups. Similar observations of variation in the value of the asymptote, referred to as $i_{max.}$, have been made in diabetic (18) and in thiamine-deficient (19) animals, and also in animals fed lactate in place of glucose (20). In each of these cases, $i_{max.}$ was higher than in the normal control.

Fatty Acids—The rises in deuterium concentration observed in the fatty acids both of liver and of carcass show certain common characteristics (*cf.* Figs. 4 and 5). In every case the thyroid-fed animals gave samples richer in deuterium than did those of the other two groups. From an inspection of the curves it appears that in the thyroid-fed animals the isotope concentrations approach a maximal value of about 45 to 50 atoms per cent D. On the other hand, in the cases of the normal controls and thiouracil-treated animals, an $i_{max.}$ of about 35 atoms per cent D is approached. Estimation of the half life times in the case of the liver fatty acids leads to a value of about 1 day when thyroid was administered, and about 1½ days for the normal and thiouracil-treated groups. As expected, the fatty acids of the carcasses in each group of animals approached the same maximal isotope concentrations as those of the livers, but at a lower rate. In the case of the carcass fatty acids, half the maximal isotope concentration is reached in about 2½ days under thyroid administration and in about 4½ days in the other two groups. With use of these figures together with the quantities of depot fatty acids recovered from each of the three groups of animals (Table II), it has been estimated that 1.2, 1.1, and 1.0 gm. of depot fatty acids per rat

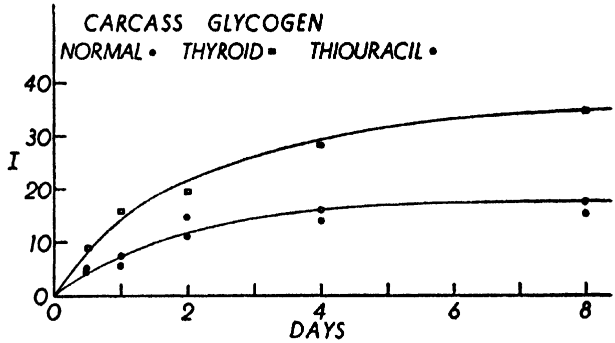

FIG. 3. Deuterium concentrations in carcass glycogen samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

were replaced daily by newly synthesized fatty acids in the normal, the hypothyroid, and the thyrotoxic animals, respectively.

The more rapid rise in deuterium concentration of the fatty acids of the carcasses of the rats receiving thyroid substance is thus ascribable to the higher value of $i_{max.}$ and the paucity of depot fat. The diminutions in

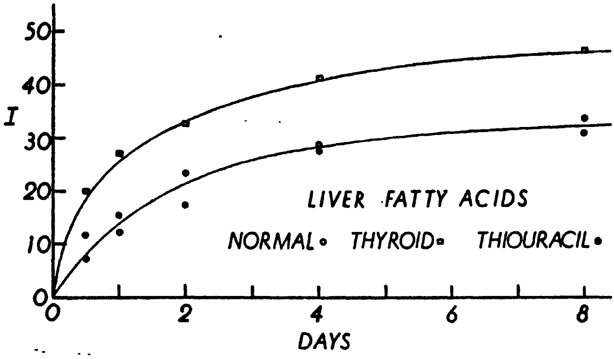

FIG. 4. Deuterium concentrations in liver fatty acid samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

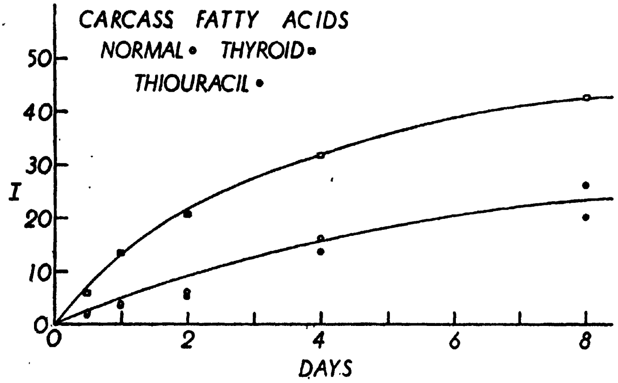

FIG. 5. Deuterium concentrations in carcass fatty acid samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

the quantity of depot fat which followed the administration of thyroid gland, in spite of a virtually normal rate of deposition of newly synthesized fatty acids, must therefore be attributed to accelerated degradation of body fat. This is in contrast to the mechanism of diminution in the quantity of body fat observed in diabetes (19), undernutrition, and thiamine deficiency (20). In all of these cases, a striking diminution in the rate of lipogenesis was observed.

Sterols—Whereas the level of blood cholesterol, both in man (21) and in experimental animals (22), is known to be influenced by the activity of the thyroid gland, no significant variations in the quantities of sterol recovered from the livers and carcasses of the three groups of animals here studied were noted (Table II). Consideration of the concentrations of deuterium found in the sterol digitonides of the livers of the three groups of rats reveals the same tendency that has previously been noted in the other tissue constituents (Fig. 6). The isotope concentrations in the materials from the rats receiving thyroid gland are higher than normal, while the thiouracil-treated rats appear to give slightly lower values. However, the quantitative differences here are not as striking as with the glycogen and fatty acids. The curves for the liver sterols proper, corrected for the presence of digitonin (23), appear to approach

TABLE II

Average Weights of Tissue Constituents

Liver constituents are reported as per cent of wet liver weight, carcass constituents as per cent of total body weight.

	Normal	Thiouracil	Thyroid
	per cent	*per cent*	*per cent*
Liver glycogen	3.26	3.90	0.00
Carcass glycogen	0.148	0.109	0.107
Liver fatty acids	2.04	2.04	2.40
Carcass fatty acids	5.28	5.79	4.00
Liver sterol digitonides	0.72	0.69	0.68
Carcass sterol digitonides	0.48	0.51	0.57
Liver "protein"	16.1	13.3	17.4

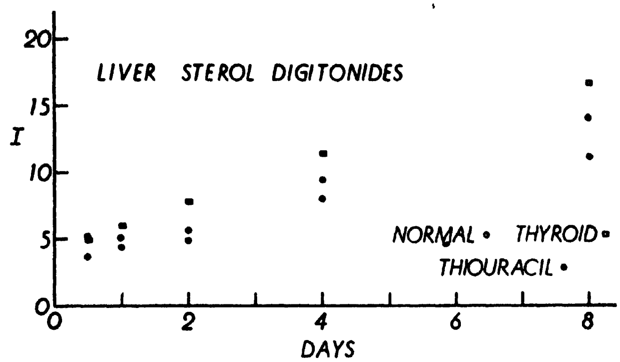

FIG. 6. Deuterium concentrations in liver sterol digitonide samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

maximal values between 45 and 60 atoms per cent D. A half life of 3 to 4 days is here estimated.

In view of the relatively long half life of carcass sterols (2), the low deuterium analyses obtained on the carcass sterol digitonides are not surprising. The resultant higher per cent analytical error would in part account for the wide scattering of these points (Fig. 7), which precludes any satisfactory interpretation.

Liver "Protein"—The isotope concentrations of the liver protein fractions are plotted in Fig. 8. It is recognized that the protein mixture analyzed is far from homogeneous and that the processes leading to the biological incorporation of deuterium into the protein molecule are numerous and cannot at present be exactly described. Among these an important one probably is the introduction into the proteins of deuterio-amino acids which have been synthesized in a medium of heavy water; and in so far as this is true, the rise in deuterium concentration of the liver protein should bear some relationship to the rate of protein synthesis. Study of Fig. 8 reveals a tendency toward higher isotope concentrations in the liver proteins of the thyroid-fed animals, when compared with the other two groups. The thiouracil-fed animals do not deviate significantly from the normal. Here again the differences are not marked enough to allow a quantitative comparison. Under these experimental conditions an $i_{max.}$ of between 20 and 25 atoms per cent D appears to be approached. From these figures the half life of the process which we are measuring would be 2 to 3 days.

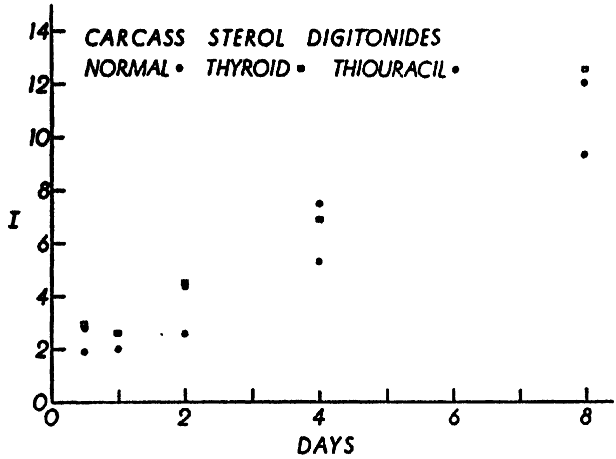

FIG. 7. Deuterium concentrations in carcass sterol digitonide samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

Summary of Results—Certain generalizations may be drawn from the results described. In virtually every case in which the data were susceptible of analysis, the compounds isolated from the rats receiving thyroid gland were richer in deuterium than the corresponding compounds isolated from the normal animals. In certain cases, the compounds isolated from the thiouracil-fed rats contained less isotope than did the corresponding compounds from the normal animals. In several cases it was possible to estimate the isotope concentration, $i_{max.}$, which would be approached in infinite time, and in each of these cases, the value was above normal when thyroid was fed. These differences accounted, in large part, for the differences in the rates of incorporation of deuterium into the various compounds studied.

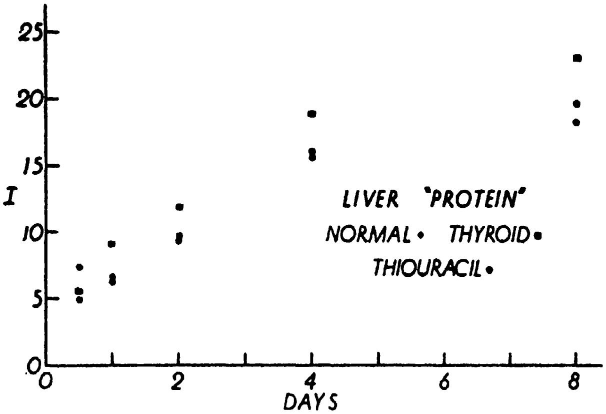

FIG. 8. Deuterium concentrations in liver "protein" samples. The deuterium concentrations, expressed as percentage of deuterium in body water, have been plotted against duration, in days, of the deuterium oxide regimen.

The implication of this finding is that, of the hydrogen stably bound in newly synthesized fatty acid and glycogen, a fraction larger than normal arises from the body water when thyroid gland is administered. A similar tendency seems to exist in the case of sterols and protein. This would suggest that the pool of smaller molecules which serve as common precursors in the biosynthesis of all these larger molecules is richer than normal in deuterium in hyperthyroid states. Such a situation could arise if a larger proportion of the fundamental building blocks were derived from the breakdown of isotopic body constituents than from the non-isotopic diet. This assumption is in agreement with the well known increase in gaseous metabolism and nitrogen excretion in hyperthyroid states. The thiouracil-treated group of animals did not differ sufficiently from normal to allow conclusions to be drawn as to the hypothyroid state.

SUMMARY

The rates of incorporation of deuterium from the body water into the glycogen, fatty acids, and sterols of liver and carcass, as well as into the protein of liver, have been studied in immature rats. Normal rats, rats fed thiouracil, and rats fed thyroid gland have been compared.

The rate of rise in deuterium concentration of each constituent was greater than normal when thyroid substance was fed. A possible interpretation of these findings is given.

In contrast to other situations in which diminution in the quantity of body fat has been studied, the cachexia of hyperthyroidism has been found not to be accompanied by an interference with fatty acid synthesis.

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STUDIES ON THE CYCLOPHORASE SYSTEM

VII. D-ASPARTIC OXIDASE

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The present communication deals with the properties of an oxidase found in rabbit kidney and liver which specifically catalyzes the oxidation by molecular oxygen of D-aspartic acid to oxalacetic acid. This oxidase was discovered in the cyclophorase preparation at the 3rd residue stage, but subsequent study revealed that the bulk of the enzyme is not associated with the particulate elements and can be found in high concentration in the supernatant fluid obtained by centrifuging the first homogenate.

Results

Specificity of Enzyme—The soluble oxidase prepared as described in the experimental section catalyzes the oxidation of D-aspartate and to a much lesser degree that of D-glutamate but not that of L-aspartate or oxalacetate (*cf.* Table I). The same preparation has little if any effect on D-methionine, D-phenylalanine, DL-alanine, DL-leucine, DL-α-aminobutyric acid, DL-ornithine, D-histidine, DL-tryptophan, or DL-α-aminophenylacetic acid.

Effect of Ethyl Alcohol—When the oxidation of D-aspartate is carried out in the presence of ethyl alcohol, the rate of oxygen uptake is about doubled (*cf.* Fig. 1). Analysis of the reaction mixture disclosed that the reaction has proceeded according to the following equations (*cf.* Table II):

$$\text{Aspartate} + O_2 \longrightarrow \text{oxalacetate} + H_2O_2 + NH_3 \tag{1}$$

$$H_2O_2 + \text{alcohol} \xrightarrow{\text{Catalase}} \text{acetaldehyde} \tag{2}$$

$$\text{Oxalacetate} \longrightarrow \text{pyruvate} + CO_2 \tag{3}$$

Keilin and Hartree (1) have shown that this alcohol effect is specific for oxidase systems which form H_2O_2 in the presence of catalase. Oxalacetate is decomposed so readily by a carboxylase present in the extract that it can be demonstrated almost exclusively in the form of pyruvic acid.

Coenzyme Requirement—No soluble coenzyme has been found to be required for the activity of the D-aspartic oxidase. The nature of the prosthetic group which is apparently firmly linked to the protein has yet to be established.

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Non-Identity with Krebs D-*Amino Acid Oxidase*—Apart from the fact that the preparation of the D-aspartic oxidase has little if any action on other D-amino acids, there is the additional evidence that benzoate, which according to Klein and Kamin (2) completely inhibits the D-amino acid oxidase of Krebs in 0.003 M concentration, has no effect on the D-aspartic oxidase (*cf.* Table III). The residual D-amino acid oxidase activity present in the enzyme extract was, however, completely inhibited.

TABLE I

Specificity of D-*Aspartic Oxidase*

Substrate (50 μM)	Oxygen uptake	Ammonia production
	microatoms	*μM*
None	5.2	5.2
D-Aspartate	93.5	46.0
D-Glutamate	24.9	13.4
D-Methionine	6.0	4.8
Oxalacetate	4.8	

Each cup contained 1 cc. of the enzyme solution, 0.5 cc. of 0.1 M phosphate buffer of pH 7.3, 0.4 cc. of 4 per cent ethyl alcohol. Final volume 3 cc. Alkali in center well. Oxygen in gas phase; 38°. Time of experiment, 70 minutes.

TABLE II

Products of Reaction

DL-Aspartate added (1)	Oxygen absorbed (2)	Ammonia formed (3)	Oxalacetate formed (4)	Pyruvate formed (5)	$\frac{O}{NH_3}$	$\frac{(4) + (5)}{(3)}$	$\frac{(4) + (5)}{(2)}$
μM	*μM*	*μM*	*μM*	*μM*			
100	35.8	37.7	4.8	28.8	0.95	0.89	0.94

The experimental vessels contained 1.5 cc. of enzyme, 0.5 cc. of 0.1 M phosphate buffer of pH 7.3 and 0.4 cc. of 4 per cent ethyl alcohol. Duplicate vessels were pooled for analyses. All the values are corrected for the blank without added substrate. Time of experiment, 70 minutes.

Oxidation of D-*Aspartate in Cyclophorase System*—A significant amount of D-aspartic oxidase activity is found associated with the particulate elements of kidney or liver cyclophorase at the 3rd residue stage. Here again, there is no action on the natural isomer, L-aspartate. Approximately 1 mole of ammonia is liberated for each atom of oxygen absorbed by the cyclophorase system in the presence of DL-aspartate (*cf.* Table IV). When the oxidation of aspartate is allowed to proceed in the presence of some member of the citric acid cycle, the ratio atoms of oxygen absorbed to the moles of NH_3 liberated exceeds a value of 1 and under appropriate

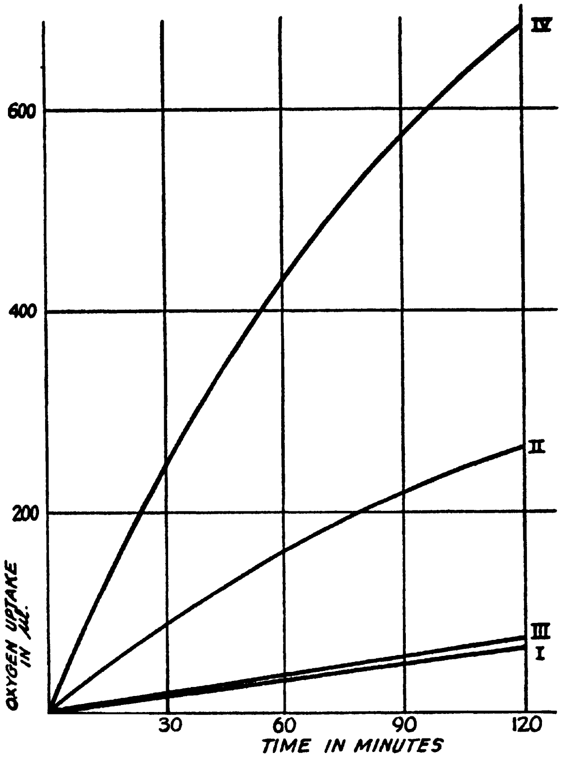

FIG. 1. Effect of ethyl alcohol on rate of oxidation of D-aspartate by D-aspartic oxidase. The complete system contained 1.0 cc. of enzyme solution, 0.5 cc. of 0.1 M phosphate buffer of pH 7.3, 0.5 cc. of 0.2 M DL-aspartate, 0.4 cc. of 4 per cent ethyl alcohol, alkali in the center well (0.2 cc.), and water to make up to a final volume of 3 cc. Oxygen in the gas space. Curve I, minus substrate and alcohol; Curve II, minus alcohol; Curve III, minus substrate; Curve IV, complete system.

TABLE III

Insensitivity of D-Aspartic Oxidase to Benzoate

Addition (50 µM)	With benzoate		Without benzoate		Inhibition by benzoate, based on ammonia values
	Oxygen uptake	Ammonia production	Oxygen uptake	Ammonia production	
	microatoms	*µM*	*microatoms*	*µM*	*per cent*
None	6.7	4.5	5.2	5.2	
D-Aspartate	91.0	45.9	93.5	46.1	0
D-Glutamate	26.0	12.0	24.9	13.4	9
D-Methionine	6.7	4.5	8.2	9.5	100

Each cup contained 1 cc. of the enzyme solution, 0.5 cc. of 0.1 M phosphate buffer of pH 7.3, 0.4 cc. of 4 per cent ethyl alcohol. Final volume 3 cc. Alkali in center well. Oxygen in gas phase; 38°. Time of experiment, 70 minutes.

conditions attains a value of 6, which corresponds to the complete oxidation of aspartate to carbon dioxide, ammonia, and water according to the following equation (*cf.* Table V).

TABLE IV

Oxidation of D-Aspartate in Kidney Cyclophorase System without Additions of Components

Additions	Oxygen uptake (1)	Ammonia production (2)	$\frac{(1)}{(2)}$ (corrected for blank)
	microatoms	*μM*	
None	7.1	1.42	
DL-Aspartate (60 μM)	21.2	13.35	1.19
DL-Methionine (60 μM)	8.8	3.97	0.6
DL-Phenylalanine (60 μM)	8.8	3.22	1.0
Benzoate (0.003 M)	5.6	2.4	
DL-Aspartate + benzoate	20.0	13.4	1.28

Each manometer cup contained 1 cc. of kidney cyclophorase system at the 3rd residue stage, 0.5 cc. of 0.1 M phosphate buffer of pH 7.3, and the additions as indicated above. Oxygen in gas phase; 38°. Time of experiment, 70 minutes.

TABLE V

Complete Oxidation of D-Aspartate in Kidney Cyclophorase System

Additions	Oxygen uptake (1)	Ammonia production (2)	$\frac{(1)}{(2)}$ (corrected for blank)
	microatoms	*μM*	
1. α-Ketoglutarate (5 μM)	64.4	4.35	
" + DL-aspartate (10 μM)	91.5	9.21	5.6
2. α-Ketoglutarate (5 μM)	62.4	5.48	
" + DL-aspartate (5 μM)	85.3	9.26	6.1

Each manometer cup contained 1 cc. of kidney enzyme at the 3rd residue stage, 0.5 cc. of 0.1 M phosphate buffer of pH 7.2, 0.3 cc. of 0.01 M adenylic acid, 0.2 cc. of 0.02 M magnesium chloride, and 0.3 cc. of 0.33 M fluoride. Oxygen in gas phase; 38°. Time of experiment, 70 minutes.

$$\text{(4)} \qquad \text{Aspartate} + 6\text{O} \rightarrow 4\text{CO}_2 + \text{NH}_3 + 2\text{H}_2\text{O}$$

Effect of Washing Cyclophorase Gel—On successive washings, the amount of D-aspartic oxidase in the cyclophorase gel is gradually reduced (*cf.* Table VI). Thus at the 6th residue stage there is found only about half the activity as at the 3rd residue stage, and in turn at the 3rd residue stage there is correspondingly less than at the 1st residue stage. Much the same applies to the small amounts of Krebs' D-amino acid oxidase found in the cyclophorase preparation. In this respect these two oxidases are unique among the other oxidases of the cyclophorase system which are not extractable by indefinite amounts of washing of the gel.

TABLE VI

Extraction of D-Aspartic Oxidase from Kidney Cyclophorase Gel

The results are expressed as microliters of oxygen per 70 minutes.

	R_3K	R_6K
D-Aspartic oxidase activity	158	70
DL-Methionine activity	20	0

The oxygen uptakes are corrected for the blanks. Each manometer cup contained 1 cc. of kidney cyclophorase system at the 3rd (R_3K) and 6th (R_6K) residue stage respectively, 0.5 cc. of 0.1 M phosphate buffer of pH 7.3, and the additions as indicated above. Oxygen in gas phase; 38°. Time of experiment, 70 minutes.

TABLE VII

Aerobic Amination of Oxalacetate

Additions	Final ammonia	Δ ammonia	Final α-amino nitrogen	Δ α-amino nitrogen
	μM	μM	μM	μM
Oxalacetate (85 μM) + ammonia (20 μM)	15.6	6.6	11.4	6.6
	13.6	8.6	12.7	7.9
As above except ammonia added at end of experiment	22.2		4.8	

Each manometer vessel contained 1 cc. of R_3K, 0.3 cc. of 0.01 M adenylic acid, 0.3 cc. of 0.1 M phosphate buffer of pH 7.2, 0.1 cc. of 0.02 M magnesium chloride, and 0.05 cc. of 0.1 M α-ketoglutarate. Time of experiment, 80 minutes. Oxygen in gas phase; 38°.

Aerobic Amination of Oxalacetate—When oxalacetate is allowed to undergo oxidation in the kidney cyclophorase system in the presence of ammonia and a catalytic amount of α-ketoglutarate, there is observed (1) disappearance of ammonia and (2) the appearance of a corresponding amount of α-amino nitrogen (*cf.* Table VII). Since the substance formed in this reaction is not glutamate, it seems reasonable to assume that D-aspartate is formed according to the equation

$$(5) \qquad 3\ \text{Oxalacetate} + NH_3 \xrightarrow{O_2} \text{aspartate} + \text{citrate} + CO_2$$

Under the conditions of the experiment citrate will undergo further oxidation to carbon dioxide and water. As long as there is excess of oxalacetate and ammonia, aspartate will continue to accumulate. In another communication it will be shown that an analogous aerobic synthesis of glutamate ensues in the presence of excess α-ketoglutarate and ammonia. In this instance L-glutamate can be identified by a specific method.

Oxidation of L-*Aspartate*—Many experiments have been carried out to determine whether or not L-aspartate can be oxidized in the cyclophorase system, but all have been consistently negative despite the fact that there is present some glutamic-aspartic transaminase which theoretically should permit the oxidation of L-aspartate to proceed in the presence of α-ketoglutarate. It must be concluded that this transaminase does not operate efficiently at the low levels of α-ketoglutarate which exist during the operation of the citric acid cycle.

EXPERIMENTAL

Preparation of Enzyme—Rabbit kidney homogenate, prepared as described by Green *et al.* (3), is centrifuged in the cold. The supernatant fluid is brought to pH 5.4 by addition of acetic acid, and the precipitate is removed and discarded. The supernatant fluid is neutralized to pH 7.0 and mixed with 6 volumes of cold acetone. The precipitate is washed with acetone and dried in air. The enzyme can be extracted from the acetone powder with 10 parts of water or 0.1 M phosphate buffer of pH 7.2. The insoluble residue does not retain any of the activity. Such extracts are stable for an indefinite period when kept frozen at −20°.

Methods—Ammonia was estimated by microtitration after distillation from tungstic acid filtrates in a Markham distillation apparatus (4). Oxalacetic and pyruvic acids were estimated by the method of Straub (5), and α-amino nitrogen by the method of Moore and Stein (6). Glutamic acid was estimated by means of a specific bacterial carboxylase (7).

This investigation was aided by a grant from the Commonwealth Fund. We are indebted to Mrs. Betty Noyce for her assistance with some of the early experiments, to Dr. Jesse Greenstein for a sample of D-glutamic acid, and to Dr. R. H. Burris for dried preparations of glutamic decarboxylase.

SUMMARY

A soluble D-aspartic oxidase has been obtained from rabbit kidney and liver which catalyzes the aerobic oxidation of aspartate to oxalacetate and NH_3. Hydrogen peroxide is formed in the reaction. In cyclophorase preparations containing the D-aspartic oxidase, D-aspartate undergoes complete oxidation to carbon dioxide and ammonia.

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THE UTILIZATION OF ACETATE FOR THE SYNTHESIS OF FATTY ACIDS, CHOLESTEROL, AND PROTOPORPHYRIN*

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We have previously shown that acetic acid is used in the rat and mouse for the synthesis of fatty acids (1), cholesterol (2), and hemin (3). Since these experiments were all of relatively short duration, it was not possible to do more than to estimate roughly the fractions of these substances which were derived from acetic acid. We have, therefore, carried out experiments in which sodium deuterioacetate was administered to growing rats for a period sufficiently long to insure that the isotope concentration of the fatty acids, cholesterol, and hemin will have attained a maximum value. The data give a lower limit for the fraction of each of these compounds derived from acetic acid. For the fatty acids and cholesterol these fractions are 0.20 and 0.45 respectively. Approximately these values have been obtained by Anker (4).

EXPERIMENTAL

Sodium deuterioacetate was prepared by the method previously described (3). It contained 25.0 atom per cent excess deuterium. Five rats, each weighing about 28 gm., were kept in separate metabolism cages and given weighed amounts of the following diet: 740 gm. of cerelose, 200 gm. of casein, 20 gm. of celluration, and 40 gm. of the Sure salts (5) (1000 gm. total). This diet contained 30 mg. of ether-extractable material per kilo.

To each kilo of the above diet were added the following factors: 300 mg. of *p*-aminobenzoic acid, 1 gm. of inositol, 100 mg. of nicotinamide, 2 mg. of thiamine, 4 mg. of pyridoxine, 2 mg. of vitamin K, 10 mg. of calcium pantothenate, 4 mg. of riboflavin, and 20 mg. of α-tocopherol.

Each rat received 1 drop of oleum percomorphum weekly.

Sodium deuterioacetate was added to the diet at a level of 1 mm. of sodium deuterioacetate per 100 gm. of body weight per day throughout the experimental period.

Pooled urine samples were collected and analyzed for deuterium at approximately 10 day intervals. 26 days after the start of the experiment

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the deuterium concentration in the urine attained a value of 0.035 atom per cent excess. For the remaining 132 days of this experiment the average of the deuterium concentration of the urine, determined at 10 day intervals, was 0.032 atom per cent excess.

Rat 1 was sacrificed on the 29th day of the experiment, Rat 2 on the 43rd day, Rat 3 on the 68th day, Rat 4 on the 102nd day, and Rat 5 on the 158th day. Fatty acids and cholesterol digitonide were isolated from the rat livers and hemin from blood by the usual procedures. On the 156th and 157th days of the experiment 150 mg. of β-phenyl-DL-α-aminobutyric acid were added to the diet of Rat 5. Phenyl-L-acetamidobutyric acid was isolated from the urine. It contained 0.266 atom per cent deuterium excess. The isotope concentration in the acetyl group, therefore, was 1.33 atom per cent excess. The various experimental values are given in Table I.

TABLE I

Deuterium Concentration

Rat No.	Weight at beginning of experiment	Weight at death	Length of feeding period	Liver		Hemin	Body water	Carcass fatty acid
				Fatty acids	Cholesterol			
	gm.	*gm.*	*days*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*
1	28	90	29	0.119	0.276	0.098	0.031	0.070
2	27	148	43	0.073	0.258	0.137	0.027	0.058
3	28	182	68	0.069	0.234	0.267	0.024	0.060
4	28	198	102	0.080	0.282	0.428	0.031	0.072
5	30	260	158	0.070	0.240	0.428	0.031	0.081
Average........................				0.082	0.258			0.068

DISCUSSION

As the lipide content of the diet was negligibly small, all the isolated lipides of the liver either were present in this tissue at the start of the experiment or had been synthesized during the experimental period. During the early period of the experiment the rats were growing rapidly, and presumably were increasing the total quantity of liver lipides. The turnover time for the total fatty acids being about 9 days (6), most of the fatty acids, in any case, must have been replaced by newly synthesized fatty acids. The data for the liver as well as the carcass fatty acids indicate that the maximum value was attained early in the experimental period. The average isotope concentration in the liver fatty acids was 0.082 ± 0.014 atom per cent excess, while that in the carcass fatty acids was 0.068 ± 0.007. These values are in fair agreement. In order to calculate what fraction of the liver fatty acids are derived from acetate, it is necessary to know the isotope concentration of the acetate available to the cell for synthetic purposes. We have previously shown (3) that dietary acetate is considerably diluted by acetate formed in the intermediary metabolism. When, as was the case in this experiment, 1 mole of sodium acetate is administered in the diet per day per 100 gm. of body weight, this acetate is diluted by a factor of 20. Since the administered deuterioacetate contained 25.0 atom per cent excess deuterium, the mixture of endogenous and exogenous acetate available for synthetic reactions must have been 1.25 atom per cent excess. In confirmation of this the acetyl group of acetylphenylaminobutyric acid excreted on the 156th and 157th days of the experiment contained 1.33 atom per cent excess deuterium. Nearly the same dilution factor is found after 156 days feeding of deuterioacetate as in a 3 day experiment. The maximum isotope concentration which could be expected in the fatty acids, even if acetate were the sole precursor of the fatty acids, could be only half that of the available acetate (1.33 atom per cent excess) or 0.67 atom per cent excess. The reason is that the acetyl group CH_3CO is reduced to a $—CH_2CH_2—$ group. At most one-half of the hydrogen atoms of the latter structure is derived from the acetate; the remainder comes from the water of the medium. The isotope concentration found in the liver fatty acids is but 0.082 atom per cent excess or 12 per cent of that calculated on the assumption that acetate is the sole precursor of the fatty acids. This value is a minimum one, since deuterium might have been lost in the chemical reactions which intervene between the acetate and the higher fatty acids. Indeed when we previously fed acetate labeled with C^{13} in the carboxyl group and D in the methyl group to mice (1), the C^{13} data indicated that about one-fourth of the fatty acids was synthesized from acetate, while the deuterium data yield a lower value, one-sixth. If the same reactions are involved in the synthesis of fatty acids in the rat as in the mouse, the same loss of deuterium in the course of the synthesis should occur. On this assumption, at least one-fifth of the carbon atoms of the fatty acids synthesized in the rat is derived from acetate. This value of necessity is too low, since some of the unsaturated acids are not synthesized by the rat.

Similar considerations apply to the liver cholesterol. The value found for the five rats cluster about an average value of 0.258 atom per cent excess. Clearly in each animal the liver cholesterol has been completely regenerated during the experimental period. Since the oxygen of the —CO group of the acetate would here be replaced by the hydrogen of the body fluids, we would also expect that the isotope concentration in the cholesterol, if it were entirely formed by the condensation of acetic acid, would be about 0.6 atom per cent excess. The actual value found suggests that about 45 per cent of the carbon atoms of cholesterol is derived from acetic acid. This value is in agreement with the estimate we have previously made in experiments with mice.

For both the fatty acids and cholesterol we have neglected the contribution to their deuterium content made by the deuterium present in the body fluids. During the major portion of the experimental period the isotope concentration in the body fluids averaged 0.032 atom per cent excess. Its contribution to the isotope concentration of these lipides could not have been greater than 0.016 atom per cent excess (7). Graphic representation of the isotope concentrations in the hemin indicates that it reached a maximum value at about the 100th day. At this time all the porphyrin originally present in the hemoglobin of the rats had been completely replaced by newly synthesized porphyrin. The life span of the red cell of the rat must, therefore, be close to 100 days, a value strikingly similar to that found in the dog (8) and in man (9).

Because the porphyrin rings proper do not contain carbon-bound hydrogen, all the deuterium atoms must be either in the side chains or attached to the methine carbon bridges. Of these hydrogen atoms about one-third (0.428 to 1.33) must be derived from the methyl group of acetic acid. It is not possible at present to make any further deductions.

SUMMARY

Growing rats were kept on a lipide-free diet to which was added 1 mM of sodium deuterioacetate per 100 gm. of weight per day. The isotope concentrations in the fatty acids and cholesterol indicate that 20 and 45 per cent respectively of the carbon atoms of these compounds are derived from acetate. About one-third of the hydrogen atoms of hemin is derived from the hydrogen atoms of the methyl group of the acetic acid.

The life span of the erythrocyte of the rat appears to be approximately 100 days.

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THE METABOLISM OF ACETONE BY SURVIVING RAT LIVER*

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Acetone has long been considered an end-product of fat metabolism. Attempts to ascertain whether it is further metabolized in the organism have been inconclusive (1, 2). In a search for precursors of cholesterol other than acetate, we have tested acetone and have found that rat liver slices, incubated with deuterioacetone, yield deuteriocholesterol.

EXPERIMENTAL

Deuterioacetone—In a typical preparation 10 cc. of acetone were added to a solution of 100 mg. of potassium carbonate in 25 cc. of 60.5 per cent D_2O. The solution was refluxed for 6 hours and was then fractionated through a Vigreux column. The fraction boiling at 56–57° was dried over anhydrous copper sulfate. Its deuterium content was 48.3 atom per cent excess.

Deuterioacetate—Sodium deuterioacetate was prepared as described previously (3).

Incubation Experiments—The incubations were carried out as described previously (4). Livers from Wistar strain rats weighing close to 150 gm. were used throughout the experiments. A Krebs flask was charged with 30 cc. of Krebs' phosphate buffer, liver slices, and oxygen. The acetone was then introduced and the flask sealed.

After incubation for 3 hours, cholesterol was isolated as the digitonide by the usual procedure, and was analyzed for its deuterium content. The deuterium concentration in the cholesterol was obtained by multiplying the deuterium concentration of the digitonide by 3. The results are given in Table I.

DISCUSSION

These experiments establish that in the rat liver a mechanism exists for the utilization of acetone for the biosynthesis of cholesterol. Acetone apparently is not as effective a precursor as acetate, but an absolute compari-

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son is difficult for several reasons: The relative rates of diffusion into the cells, the toxicities, and the stability of the carbon-deuterium bond differ in the two metabolites. However, it is clear that acetone can be used for cholesterol synthesis.

Acetone might be utilized for the synthesis of cholesterol either by incorporation of the molecule *in toto* or after conversion to some other metabolite. That acetone is, at least partially, converted to acetate is suggested by the diminished incorporation of deuterium into cholesterol when normal acetate is added to the system together with deuterioacetone (Table I). Investigations now in progress in this laboratory demonstrate the conversion of acetone to acetate: When labeled acetone is administered to rats together with α-amino-γ-phenylbutyric acid, the acetylamino acid excreted is labeled.[1] On the other hand a direct incorporation of the entire acetone carbon chain is not excluded. That the lowering of the isotope concentration in the cholesterol when deuterioacetone and normal acetate

TABLE I

Cholesterol Synthesis from Deuterioacetone in Rat Liver Slices

Experiments having the same ordinal numbers were carried out with slices from the same livers.

Experiment No.	Acetone addition		Acetate addition		Cholesterol
		Deuterium concentration, atom per cent excess		Deuterium concentration, atom per cent excess	Atom per cent excess
	mg.		mg.		
Ia	158	29.6	0		0.144
Ib	158	29.6	0		0.144
IIa	158	48.3	0		0.204
IIb	158	48.3	0		0.201
IIIa	0		100	57.4	0.417
IIIb	158	48.3	0		0.180
IVa	0		50	57.4	0.702
IVb	94	0.0	50	57.4	0.441
Va	47	48.3	0		0.321
Vb	47	48.3	0		0.312
Vc	63	48.3	126	0.0	0.006
Vd	63	48.3	*		0.306
VIa	47	48.3	0		0.165
VIb	51	48.3	32†	0.0	0.042
VIc	55	48.3	63†	0.0	0.030
VId	63	48.3	126†	0.0	0.024

* Isotonic saline containing 90 mg. of NaCl was added.

† Sufficient water was added to make the sodium acetate isotonic.

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[1] Unpublished data of D. Price.

are incubated is a specific effect of acetate and not of the sodium ion is evidenced by the absence of an inhibition of the synthesis of deuteriocholesterol after addition of sodium chloride (see Experiment Vd).

SUMMARY

1. Deuteriocholesterol is formed on incubating surviving rat liver slices with deuterioacetone, establishing that this tissue can metabolize acetone.
2. At least part of the acetone is oxidized to acetate.

We are indebted to Miss Laura Ponticorvo for her able assistance during the course of this work.

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METABOLISM OF L-ASPARTIC ACID*

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Aspartic acid, like glutamic acid, occupies a central position in amino acid metabolism. In most tissues it takes part in the transamination reaction (1). In the liver and the kidney, where all amino acids undergo deamination, the rate of this process for aspartic acid is second only to glutamic acid (2). For these reasons, one would expect the exchange of amino nitrogen of aspartic acid with the nitrogen of the metabolic pool to be more rapid than in the case of other amino acids. This is indeed true, as the present experiment with isotopic L-aspartic acid shows.

EXPERIMENTAL

Preparation of L-Aspartic Acid Containing N^{15}—L-Aspartic acid containing N^{15} was prepared by the action of *Escherichia coli* on fumaric acid and ammonia containing N^{15}. Quastel and Woolf (3) have shown that *E. coli* catalyzes the reversible reaction, fumarate + $NH_3 \rightleftarrows$ L-aspartic acid.

E. coli was grown on 2 per cent agar in ten Roux bottles. The agar medium contained 2 per cent of Bacto-tryptone and 0.5 per cent each of glucose, sodium chloride, and concentrated yeast extract. The 2 day culture of *E. coli* was separated from the agar by gentle rocking with normal saline, filtered through gauze, centrifuged, washed, and made up to 200 ml. with normal saline.

15 gm. of fumaric acid were suspended in water, neutralized with sodium hydroxide, and the solution made up to 130 ml. 4 gm. of ammonium chloride, containing 31.8 atom per cent excess of N^{15}, were added, followed by 100 ml. of Clark and Lubs 0.05 M phosphate buffer at pH 7.4, 25 ml. of *E. coli* suspension, and 20 ml. of toluene. The mixture was placed in a suction flask, which was evacuated and filled with nitrogen. The flask was placed in an incubator at 37°. The ammonia content of the mixture fell in 24 hours to 19.2 per cent and in 48 hours to 18 per cent of the original. Apparently, an equilibrium between fumaric acid and ammonia on the one hand and aspartic acid on the other was reached in 2 days under the conditions of the experiment.

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After 2 days the mixture was boiled, centrifuged, and the precipitate washed with hot water. 60 ml. of saturated copper sulfate solution were added and the mixture was placed in an ice box overnight. The copper aspartate crystals were washed with a little cold water, suspended in 600 ml. of hot water, and decomposed with hydrogen sulfide. The filtrate was evaporated to about 150 ml. and 2 volumes of 95 per cent alcohol were added. After standing overnight, the aspartic acid crystals were filtered off, washed with alcohol, and finally with ether. Yield, 6 gm.

The nitrogen of the aspartic acid thus prepared contained 31.3 atom per cent excess N^{15} corresponding to an atomic weight of 14.32. The theoretical nitrogen content of this aspartic acid (mol. wt. 133.4) is 10.72 per cent; found, 10.86 per cent.

Rat Feeding—Three male rats weighing between 240 and 290 gm. each were placed on a stock diet containing starch 71, casein 15, yeast 5, Osborne-Mendel salt mixture 4 (4), Wesson oil 3, and cod liver oil 2 per cent. After 3 days, 0.3 gm. of isotopic aspartic acid and 0.2 gm. of sodium bicarbonate were given to each rat daily for 3 days. The aspartic acid and bicarbonate were mixed with 8 to 12 gm. of the stock diet, which was made into a dough with a little water.

The food was all eaten by the rats. Feces and urine were collected daily. The combined weights of the rats were 786 gm. before, and 739 gm. after the isotopic feeding.

Preparation of Material for Analysis—24 hours after the last isotopic feeding, the rats were killed by exsanguination under ether anesthesia. Blood was removed from the heart, some saline being used for washing, and mixed with some oxalate. Stomach and intestines were removed and washed free from their contents. Other internal organs, liver, spleen, kidney, testes, lungs, etc., were removed and treated together. The bodies were skinned, leaving the muscles and bones and brain as carcass. The four fractions, namely stomach-intestine, other internal organs, skin, and carcass, were worked up separately.

Blood—The blood with saline washing was centrifuged. Part of the cells was used for the preparation of hemin. The cells were laked with water and mixed in a Waring blendor, centrifuged, and dropped slowly into 3 volumes of glacial acetic acid at 97° containing some sodium chloride. The mixture was heated on a water bath for an hour. The hemin crystals were separated by centrifuging, washed twice with 50 per cent acetic acid, twice with water, twice with 95 per cent alcohol, and once with ether. The N^{15} concentration of hemin was determined.

The plasma, containing some saline, was treated with 3 volumes of water and 2 volumes of 10 per cent trichloroacetic acid. The protein precipitate was filtered off and washed with 5 per cent trichloroacetic acid.

The total nitrogen and the N^{15} content of the cells, the plasma protein precipitate, and the plasma filtrate were determined in the usual manner. The results are shown in Table I.

Tissues—Stomach-intestine, internal organs, and carcass were separately ground in a meat grinder and homogenized in a Waring blendor. Skin was cut into small pieces with scissors. Each group of tissues was treated with 6 volumes of 95 per cent alcohol. After 3 days, the mixture was filtered on a Büchner funnel and the tissue suspended again in a fresh portion of alcohol. After three changes of alcohol, the tissues were treated similarly twice with ether. The defatted tissues were dried in air and used for isolation and analysis, as described below.

The combined alcohol and ether extracts were concentrated *in vacuo* until most of the alcohol had been removed. The residue was taken up in chloroform plus water and the mixture was shaken and then allowed to separate. The total nitrogen and N^{15} concentration of the two layers were determined separately. The nitrogen in the aqueous layer was taken as non-protein nitrogen, while that in the chloroform layer was taken as lipoid nitrogen.

TABLE I

Distribution of Isotopes in Blood

	N^{15} concentration	Total nitrogen	Total N^{15}
	atom per cent excess	*m.eq.*	*m.eq.*
Plasma non-protein N	0.830	0.57	0.005
" protein	0.598	7.85	0.047
Red cells, protein	0.086	46.4	0.040
" " hemin	0.038		

From the aqueous layer, some colorless crystals deposited on standing. These were identified as creatine hydrate by crystalline form and nitrogen content (28.0 per cent). The substance was recrystallized from water with the addition of alcohol and used for N^{15} determination.

Stomach-Intestine—The entire stomach-intestine preparation was used for the extraction of nucleic acid by the method of Hammarsten (5). The yield was about 90 mg. from 5 gm. of dried tissue. It was analyzed for N^{15}. Part of the nucleic acid was hydrolyzed and the purine precipitated by the method of Graff and Maculla (6). The precipitate and the filtrate, which should contain the pyrimidines, were analyzed for total nitrogen and N^{15}. From 21.2 mg. of nucleic acid, 1.79 mg. of nitrogen in purine precipitate and 1.17 mg. of nitrogen in purine filtrate (total 2.96 mg. of nitrogen) were obtained. The ratio of purine nitrogen to pyrimidine nitrogen is about 3:2, but the total nitrogen (14 per cent) is lower than that (18.5 per cent) calculated for desoxyribonucleic acid ($C_{29}H_{51}N_{15}P_4O_{25}$).

The protein remaining after the extraction of nucleic acid was washed with hot 5 per cent trichloroacetic acid until free from sulfate and ammonia. The washed residue was dried and used for nitrogen and N^{15} determinations.

Internal Organs, Carcass, and Skin—The dried tissues were hydrolyzed with hydrochloric acid in the usual manner and the nitrogen and N^{15} contents determined. The hydrolysates were treated with cuprous oxide according to the suggestion of Bailey *et al.* (7).

Tyrosine crystals separated from all the hydrolysates and were filtered off and recrystallized. From the filtrates of internal organs and carcass, aspartic acid and glutamic acid were precipitated as barium salts. Aspartic acid was isolated as copper salt and the glutamic acid as hydrochloride in the usual manner. Lysine was isolated from the carcass hydrolysate as benzoyllysine by the method of Kurtz (8). Arginine was isolated from the internal organs and skin as monoflavianate. This was decomposed with concentrated hydrochloric acid, the liberated flavianic acid was filtered off, and the filtrate was diluted with water, boiled with norit to remove the last trace of flavianic acid. The filtrate was evaporated to dryness *in vacuo*. The residue was taken up in alcohol and an excess of pyridine added. Arginine monochloride crystallized on standing. This was recrystallized by dissolving in a little water and adding alcohol to a concentration of 80 per cent.

The amidine and ornithine nitrogen in arginine were determined separately by the method of Barnes and Schoenheimer (9). Arginine monochloride (17.3 mg.) from the internal organs was dissolved in a baryta solution (containing 5 gm. of $Ba(OH)_2 \cdot 8H_2O$ in 30 m. of water), boiled gently under a reflux, and the liberated ammonia blown by a stream of nitrogen into a known amount of standard acid. After 21 hours, the acid solution was titrated and the N^{15} determined. The baryta solution was treated with an excess of sulfuric acid, filtered, washed, and the filtrate analyzed for total nitrogen and N^{15}. The result of nitrogen determinations was as follows: amidine nitrogen 2.20 mg. and ornithine nitrogen 2.36 mg. (total nitrogen 4.56), which amounts to 26.3 per cent; theoretical, 26.8 per cent. The experiment with skin arginine gave similar results.

A nucleic acid extract of the internal organs was prepared by Schneider's method (10) and analyzed for N^{15}. The hydrolysate of the carcass was analyzed for amide nitrogen.

The results of N^{15} analysis of the tissue components are shown in Table II.

Urine and Feces—The combined feces were ground, digested with concentrated sulfuric acid, and made up to 200 ml. Aliquot portions were taken for total nitrogen and N^{15} determinations. About nine-tenths of the combined urine plus washing was used for the isolation of allantoin by the method of Shaffer and Greenbaum (11). The allantoin was recrystallized from hot water and analyzed for N^{15}.

TABLE II

Distribution of Isotopes in Organ Components; Concentration in Atom Per Cent Excess

	Whole body	Stomach-intestine	Internal organs	Carcass	Skin
Non-protein N	0.359				
Lipoid N	0.134				
Protein N		0.541*	0.379	0.121	0.109
Amide N				0.125	
Creatine	0.062				
Tyrosine			0.155	0.044	0.034
Aspartic acid			0.618	0.175	
Glutamic "			0.753	0.258	
Lysine				0.008	
Arginine			0.534		0.078
Amidine			0.595		0.072
Ornithine			0.480		0.085
Nucleic acid (Schneider)			0.226		
" " (Hammarsten)		0.785			
Purine ppt		0.706			
" " filtrate		0.628			

* Exclusive of nucleic acid.

TABLE III

Distribution of Isotopes in Excreta

	N^{15} concentration	Total N	Total N^{15}
	atom per cent excess	*m.eq.*	*m.eq.*
Feces	0.440	22.4	0.10
Urine	2.19	125.5	2.76
Urea	2.13	111.4	
Ammonia	1.25	2.5	
Allantoin	0.275		

With the remainder of the urine, total nitrogen, urea nitrogen, ammonia nitrogen, and the corresponding N^{15} contents were determined. The results are shown in Table III.

DISCUSSION

Recovery of Isotope—During the feeding period the rats consumed 2700 mg. of aspartic acid containing 6.34 m.eq. of N^{15}. About 90 per cent of the isotope was accounted for in the fractions analyzed (Table IV). The remaining 10 per cent must be lost in the gastrointestinal content which was washed away.

It will be noted that 43.4 per cent of the isotope was excreted and 45.5 per cent retained in the animal body. Of this 45.5 per cent, 39.4 per cent was in the proteins, while only 6.1 per cent was in non-protein and lipoid constituents.

Urinary Ammonia and Urea—The relative concentrations of N^{15} in urinary ammonia and urea after feeding an isotopic nitrogenous substance depend not only upon the nature of the substance but also upon the rate of feeding and the interval of time during which the urine is collected.

TABLE IV

Distribution of Isotopes in Excreta and Animal Body

	Total N^{15} in fraction		Per cent recovery	
	m.eq.	*m.eq.*		
Feces		0.10	1.6	
Urine		2.76	43.4	
Non-protein N		0.35	5.5	45.5
Lipoid N		0.04	0.6	
Total protein N		2.50	39.4	
Red cells	0.040			
Plasma	0.047			
Stomach-intestine	0.292			
Internal organs	0.378			
Carcass	1.200			
Skin	0.542			
Total recovery		5.75	90.5	
Unaccounted for		0.60	9.5	

After a single feeding of an isotopic amino acid with N^{15} in the α-amino group, there is a period when its concentration in blood is greater than that in the tissues because it must enter the blood before it can reach the tissues. Since urinary ammonia can be formed in the kidney directly from amino acid brought to it by the blood, the rate of this ammonia formation must depend upon the concentration of the amino acid in the blood. Urea formation, on the contrary, will depend upon the concentration of the amino acid in the liver. If this view is correct, the isotope concentration of the ammonia formed shortly after feeding should be greater than that of the urea; the ratio, N^{15} concentration in NH_3 to N^{15} concentration in urea, should be high. This is shown by human experiments[1] in which urine samples were collected at short intervals after a single feeding of aspartic acid.

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[1] To be reported in a later paper.

As more and more of the isotopic amino acid enters the liver and other tissues, two chief reactions can occur: (1) transamination and (2) oxidative deamination and subsequent urea formation. The isotopic amino acid formed by transamination and the original amino acid fed pass back and forth between the body proteins and the metabolic pool as equilibrium conditions may require. In the liver, both original and transaminated isotopic amino acids can yield their nitrogen for urea formation. In the kidneys, both can form ammonia. The ratio, N^{15} concentration in NH_3 to N^{15} concentration in urea, in the urine collected 3 to 12 hours after a single feeding will depend primarily upon the relative rates of the redistribution of the amino group of the fed amino acid among the other amino acids and of urea formation. Under uniform conditions, this ratio should be characteristic of the amino acid fed.

With continuous feeding, as in the present experiment with rats, the total isotopic substance enters the blood in smaller amounts over a longer period of time than in the case of single feeding. Consequently, ammonia formation in the kidneys from the isotopic amino acid fed will be sustained, and the ratio, N^{15} concentration in NH_3 to N^{15} concentration in urea, for the total urine over the entire feeding period will be higher than in the case of single feeding.

When the isotopic substance is mixed with food to which the rats have constant access, difference in habit of feeding may conceivably cause some variations in the ratio, N^{15} concentration in NH_3 to N^{15} concentration in urea, in different experiments. Nevertheless, the results obtained under uniform conditions should be comparable.

The ratio, N^{15} concentration in NH_3 to N^{15} concentration in urea, for aspartic acid in the present experiment is 0.59. Previous experiments with other natural amino acids with N^{15} in the α-amino group have given values of 1.60 for L-leucine (12), 1.08 for L-lysine (13), and 0.96 to 1.2 for glycine (14, 15). For racemic amino acids, the values are much higher (16, 17). For ammonium citrate, the ratio of 0.38 has been reported (18).

As a source of urinary ammonia and urea, L-aspartic acid behaves like ammonia and unlike other natural amino acids so far studied in this laboratory. This finding suggests that the amino group of L-aspartic acid is more rapidly removed to form urea than its deamination to form ammonia in the kidneys, either directly or through glutamine (19).

Whether the amino group is actually first deaminated to give ammonia which then forms urea, or whether it gives rise directly to urea, it is impossible to decide at present. It is interesting to note in this connection that a mechanism exists for the direct formation of urea from aspartic acid.

Ratner (20) has prepared a soluble enzyme system from beef liver which catalyzes the formation of arginine from citrulline, aspartic acid, and L-phosphoglyceric acid. If this is an important mechanism for the formation of urea in the intact rat, a high isotope concentration in the urea relative to the ammonia might be expected.

Distribution of Isotope in Proteins—The general pattern of distribution of N^{15} in various organ proteins after feeding isotopic aspartic acid is about the same as after feeding with other amino acids (see Table IV). These concentrations reflect the rate of turnover of the proteins of the various organs.

Distribution of Isotope in Protein Components—As in previous experiments in this laboratory with labeled amino acids, the N^{15} concentration in any component follows the same order as the N^{15} concentration in the total protein of various organs. Thus, the N^{15} concentrations of tyrosine isolated from internal organs, carcass, and skin are 0.155, 0.044, and 0.034 atom per cent excess respectively, while the corresponding concentrations for the total proteins are 0.379, 0.121, and 0.109 respectively.

In previous experiments in this laboratory, the protein component that had the highest N^{15} concentration was the amino acid fed. Glutamic acid usually came next, followed by aspartic acid. In the present experiment glutamic acid had the highest concentration of N^{15}, although aspartic acid was the amino acid fed. This is similar to the finding after feeding isotopic ammonia (21, 22) and is in harmony with the hypothesis that the amino group of aspartic acid behaves like ammonia.

The high N^{15} content of amidine nitrogen of arginine from internal organs is to be expected in view of the part arginine plays in the urea formation in the liver. Whereas in the internal organs, which include the liver, the amidine nitrogen had a higher N^{15} concentration than the total protein, this is not true for the skin, which is not a site of urea formation.

The negligible N^{15} content of lysine is to be expected, as this amino acid, considered as an essential amino acid in nutrition, cannot be synthesized in the body.

The high concentration of N^{15} in nucleic acid of stomach-intestine suggests that it has a high rate of turnover. This is noteworthy, especially as the N^{15} concentration of nucleic acid of stomach-intestine is higher than that of the total protein, while in the other internal organs the reverse is the case. This point, however, requires further investigation.

The nucleic acid of internal organs has about the same N^{15} concentration as the allantoin in the urine. This is to be expected, as the allantoin is derived from the purines.

The low N^{15} contents of creatine and hemin found in the present experiment are in general agreement with previous findings (23, 24) after

SUMMARY

1. L-Aspartic acid was synthesized from fumaric acid and ammonia containing N^{15} in the presence of *Escherichia coli*.

2. The isotopic aspartic acid was added to the stock diet of three rats for 3 days. Only 1.6 per cent of the N^{15} was excreted in the feces and 43.4 per cent in the urine, and 45.5 per cent was retained in the body.

3. The concentration of N^{15} was the highest in the plasma proteins, and least in the red cell protein, with stomach-intestine, internal organs, carcass, and skin occupying the intermediate positions.

4. The N^{15} content of urinary ammonia was lower than that of urea.

5. The N^{15} concentration of aspartic acid was lower than that of glutamic acid isolated from the same organs.

6. These last two findings together suggest that aspartic acid is so rapidly deaminated that its amino group behaves metabolically like ammonia.

We are indebted to Mr. I. Sucher for the N^{15} determinations, to Miss Laura Ponticorvo and Miss Martha Masako Yamasaki for technical assistance, and to Dr. D. Shemin and Dr. G. L. Foster for their kindly interest and helpful suggestions.

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ELECTRON MICROSCOPY OF FIBRINOGEN AND FIBRIN

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PLATE 3

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It has been demonstrated through electron microscopy and x-ray diffraction that there are present, in certain protein fibrils, structural periodicities having dimensions up to several hundred angstrom units. Examples are collagen (1–3), paramyosin (4, 5), the trichocysts of *Paramecium* (6, 7), and fibrin (8). The application of phosphotungstic acid (4) and other heavy metal compounds greatly enhances the visibility of the structures in electron micrographs and often makes structural variations apparent which are not perceptible in the unstained fibrils. It has not been established definitely whether staining involves a selective chemical reaction. However, since metal shadowing reveals in most cases that the fibril surface is corrugated with the high points corresponding to the highly staining region, it seems probable that stain deposits according to the local concentration of protein. The regions which stain more lightly, appearing as slight depressions on shadow-casting, probably represent portions which have shrunk during drying. Little, if anything, is known concerning the genesis of these macro periods or the reason for their occurrence in protein fibers. The investigation to be described was aimed at a clarification of these phenomena and is confined to fibrin and the precursor, fibrinogen.

Microscope Techniques

All micrographs were made with an RCA type B microscope which has a self-biased gun, compensated lens (9), no physical objective aperture, and is operated at 65,000 volts accelerating potential. Although space does not permit a discussion of the physical aspects of electron microscopy, it is pertinent to note that concurrent studies were made of the structure of specimen supports, the structure of metals used for shadow-casting (10), afocal imaging phenomena, and the effects of lens asymmetries on the image. If, for example, the objective lens is astigmatic, images may exhibit spurious asymmetries, giving the false impression of fibrosity in the object. As an aid to interpretation and the recognition of possible image defects, through focus series were recorded. Uranium, platinum, and nickel have a suitably fine structure for shadow-casting *when deposited in thin enough layers.* Uranium gives excellent results in estimated thicknesses of 3 to 6 A in the plane of the film. Nickel has a remarkably fine structure (11, 12) and is useful in thicknesses of less than 10 A. Shadow angles were 3:1 or 4:1.

Observations on Fibrin

Specimens of bovine fibrin for electron microscopy were prepared in the following manner: Bovine Fraction I (Armour) and thrombin (Seegers,[1] 640 units per mg.) were dissolved separately in 0.9 per cent NaCl buffered to about pH 6.5 with KH_2PO_4 buffer solution. Concentrations of Fraction I (including citrate) in solution varied from 0.5 to 2 mg. per cc., while concentrations of thrombin were of the order of 10 units per cc. Portions of the two solutions, frequently of the order of 1:1, were thoroughly mixed in a test-tube and a drop was placed immediately on a standard specimen screen with collodion or SiO supporting film. The development of the clot in the test-tube can be followed by eye. After varying times (30 seconds and up) according to the experiment, the excess solution was washed from the specimen screen with salt solution, stained with phospho-12-tungstic acid, washed with twice distilled water, and dried. The concentration of phospho-12-tungstic acid (Anachemia, Ltd., Montreal) was usually 0.1 per cent and sometimes 1 per cent. Usually it was unbuffered, but in some experiments the pH was raised to about 5.4 with potassium acid phthalate buffer. In one experiment, sodium citrate buffer was used (pH 6.5) and, in others, the clot was formed in unbuffered 1 per cent NaCl, pH 5.8.

The electron microscope observations of stained fibrils from bovine fibrin clots confirm the observations of Hawn and Porter (8) on the existence and approximate dimensions of the axial macro period. Measurements of over 200 fibrils from several preparations yield an average of 227 A. All measurements in the group were between 190 and 270 A with over 80 per cent in the range 210 to 240 A. The spacings along individual fibrils are remarkably constant (to the order of 2 or 3 per cent) compared to variations between separate fibrils. Variations as high as 20 per cent of the mean were observed between fibrils in a single specimen. No explanation can be given for the variation from one fibril to another or for the sometimes significant variations in average spacing from one preparation to another. Since the fibrils were formed on the specimen screen, tensions due to manipulation were kept to a minimum. Tensions caused by drying might be a factor, but since the fibrils were well supported and the supporting films did not break, it does not seem likely that random variations of the order of 20 per cent could be accounted for in this way. It is clear, however, that the structure is not rigid.

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[1] The author is indebted to Dr. Walter H. Seegers, Wayne University, Michigan, for the sample of thrombin used in this work.

As shown in Fig. 1, the axial macro period consists of a series of highly staining and sharply delimited bands, designated *A*, which are about 70 A wide and are separated by a region of lower scattering power about 160 A wide. In addition to the structure previously reported, there is a finer stained band (designated as *B* in Fig. 1), about 30 A wide, mid-way between the prominent bands.

Fibrin fibrils clotted from a sample of highly purified human fibrinogen[2] (Fig. 2) show a stained structure which is indistinguishable from that observed in fibrin from bovine Fraction I. The average axial spacing from a number of plates was 214 A, which is in the range of values found for the bovine material.

When the image quality is favorable, substructure is visible within the stained bands, giving the fibrils, ultimately, a particulate appearance which may be discernible in the micrograph in Fig. 2. The particles are of the order of 50 A and less in diameter and are not well resolved. In such preparations there is always a background of finer fibrin fibrils of indistinct structure approaching in dimensions the resolution limit (or to be more precise, the visibility limit) of the microscope. These finer fibrils are about 200 A and less in diameter and do not possess the characteristic striated appearance. Similarly, the striation gradually dissolves into a more or less randomly particulate structure toward tapered ends where the width is about 200 A and less. It is concluded that the absence of well defined striations under these circumstances is not due to a lack of resolution, but represents an actual disorder in the structure. The effect may be seen in Fig. 2 where the fibril parted from the bundle toward the lower right is not clearly striated. Local regions of stain concentration are visible which tend to form a vague striation in a few places, but the structure is disordered compared to that in the thick bundle. The sharpness of the stained bands increases noticeably with fibril width. Also apparent in Fig. 2 is the tendency of striations to align between adjacent fibrils in a bundle, as was noted by Hawn and Porter (8). Throughout the present investigation, this coincidence was observed with such high frequency and with such high precision that it is deemed significant. The effect seems to indicate the presence, at some stage during fiber formation, of lateral attractive forces which are at an optimum when there is lateral coincidence of like bands.

Some specimens containing well formed fibrils such as those shown in Figs. 1 and 2 were shadow-cast. The observations show that the stained regions, both *A* and *B* bands, represent slight elevations.

[2] The sample, kindly provided by Dr. J. T. Edsall of the Harvard Medical School, was from Run 183, Fraction I-2A and was 94 per cent clottable when prepared in 1945.

Observations on Fibrinogen

Specimens from bovine Fraction I and human fibrinogen were prepared by placing a drop of the material in 1 per cent NaCl on a collodion or SiO film and drawing off the excess by touching a piece of filter paper to the edge of the screen. In some experiments, a fixative such as formalin or phosphotungstic acid was applied before drying and, in others, the specimen was allowed to dry without fixation. In any case, the final treatment was a wash in twice distilled water to remove salt and citrate, after which the specimens were shadow-cast along with control films. The variations in these procedures seemed not to produce any marked differences in the observed structure of the protein layer adhering to the film. The concentration of the sample was varied over a wide range, from about 2 mg. per cc. down to about 0.001 mg. per cc. The original intention was to secure a distribution of isolated particles over the surface of the film, but difficulty was encountered in securing this condition, because the protein tends to deposit in clumps. Also, the necessary dilution factor is so great that the effect of impurities in reagents and water becomes a consideration. Furthermore, although the film structure is relatively fine, it does complicate the interpretation. Consequently, specimens made with very high dilution factors were not such as to invite confidence. On the other hand, specimens made from concentrations which resulted in a covered supporting film leave something to be desired, because the fibrinogen particles are intermingled. With the latter condition, however, it can be concluded with some confidence that the observed structure is mainly due to the protein deposited from the original sample and the problem is to resolve the elements from the mass.

In the shadowing procedure, control films were placed beside the fibrinogen preparations. Fig. 3 shows a small, typical portion of an electron micrograph made from bovine Fraction I. There is a rather confusing aggregation of particles among which numerous filamentous elements may be distinguished. In Fig. 4 are shown micrographs of a collodion control film (*a*) which had been washed with twice distilled water and a micrograph of a specimen made from the sample of human fibrinogen (*b*). The control film exhibits a structure which is about the coarsest of any recorded; yet there is no mistaking the difference between it and the fibrinogen sample. The fibrinogen completely covers the film and although it consists of a somewhat confusing aggregation, it may be seen to contain filamentous elements similar to those from the bovine Fraction I shown in Fig. 3.

In Fig. 5, *b* is shown a thick layer from bovine Fraction I shadowed very lightly with nickel in order to present the fine detail within the filaments to better advantage than is the case in either Figs. 3 or 4. Filamentous elements may be distinguished, but they are seen to be non-uniform in thickness, appearing somewhat like a string of beads. This type of structure is characteristic of high resolution micrographs of fibrinogen preparations when they have been shadowed with a suitably thin layer of metal. It is concluded that the nodose structure of fibrinogen filaments as indicated in the electron micrographs is significant.

Measurements were made of filaments which could be discerned in micrographs similar to those shown. The lengths varied from 200 to 1100 A with 85 per cent between 300 and 800 A. The average was about 600 A. Widths, which cannot be determined with high accuracy, are estimated to be in the range 30 to 40 A across the wider portions. The observations demonstrate the presence in fibrinogen preparations of asymmetric elements in accordance with the conclusions reached from physicochemical methods, and the estimated widths are not significantly different from what would be expected from experiments on double refraction of flow (13). The correlation is unsatisfactory, however, to the extent that the electron microscope observations fail to indicate a unique length, even though there are distinguishable many filaments with lengths very close to the 700 A predicted. (The filaments which are marked in Figs. 3, 4, and 5 are close to 700 A in length.) In view of the difficulty of identifying continuous filaments and locating their ends in electron micrographs, the fault lies quite possibly, but not necessarily, with the electron microscope observations. It would be desirable to improve the electron microscope technique so that a fair sample of isolated, identifiable elements could be measured with certainty before concluding that the two methods are inconsistent. In any event, there is no unequivocal relation between the lengths of fibrinogen filaments as determined by either method and the dimensions of the macro period as seen with the electron microscope.

Formation of Fibrin

The absence of correlation between lengths of the precursor particles and the magnitude of the striations in fibrin fibrils indicates that fiber formation is not simply a matter of assembling semirigid units in the manner of crystal growth as would be suggested by the extreme regularity of the final structure. Some attention was therefore given to a study of fibers during formation through the use of short clotting times and decreasing amounts of thrombin, with a view to obtaining evidence concerning the manner in which fibrinogen filaments associate. It has been noted that with the ultimate resolution stained fibrin appears particulate and the stained bands appear to represent regions where there is a relatively high concentration of stained particles. It is not likely that the particles represent elementary units. They are probably aggregates of smaller, unresolved elements. This appearance is consistent with the particulate structure observed in fibrinogen filaments as shown in Fig. 5. When the thrombin concentration was decreased so that the clot formed very slowly, most of the fibrils showed a randomly particulate structure and little if any evidence of striations. Often, a fibril would be striated in some portions and unstriated in others. In the transition regions, the impression is gained that the occurrence of striations results from local concentrations of particles which are visible, but randomly disposed, in the unstriated portions. Although there is no marked longitudinal structure in such stained fibrils, shadow-casting reveals longitudinal filaments, indicating that formation of fibrin involves mostly lateral association of fibrinogen filaments. The observations suggest that in the clotting process the fibrinogen filaments associate laterally, after which there is a readjustment of the constituents producing variations of protein concentration along the fiber axis. The regions of higher concentration appear relatively dense after staining or as elevations when the dried material is shadow-cast.

It is to be noted that fibrils sometimes formed in solutions as they stood without any thrombin being added (some thrombin may be present as a trace in the fibrinogen material), but such fibrils have not been observed to be striated. Similarly, fibrils formed with very low thrombin concentration were mostly unstriated.

DISCUSSION

No satisfactory theory has yet been developed to account for macro periods of the sort described. It has been proposed by Astbury (14, 15) that the large periods are directly related to the much smaller dimensions of polypeptide chains as determined by x-ray analysis. According to this proposal, the macro periods represent the extent along the fiber axis of a single chain molecule in specific configuration. The long spacings should therefore be an integral multiple of certain of the short spacings. Some of the defects of the theory from the x-ray standpoint have been discussed by Bear (2). A serious difficulty arises from the fact that experimentally the short spacings are singularly unaffected by changes in dimensions of the long spacings. The electron microscope results indicate that the macro periods are associated with the distribution of particles having dimensions between those of the short and the long spacings. Structural distinctions visible in electron micrographs may possibly represent significant structural differences on the polypeptide level of dimensions, but before such a correlation could be made, it would be desirable to have an estimate of the size of the diffracting regions which produce the wide angle pattern. Wide angle patterns, remarkably similar to those for certain other fibrous proteins, have been shown for fibrinogen and fibrin (16), but no long spacings have been reported for either. The inhomogeneities which are apparent in electron micrographs of both of these substances suggest that the diffracting regions producing the wide angle pattern are probably quite small, possibly in the range of 50 A and less.

It must be acknowledged that the 700 A length of fibrinogen filaments as determined from flow birefringence data (13) is quite close to 3 times the average fibrin spacing as reported here. The possibility therefore suggests itself that the fibrinogen filament contains three preformed macro periods and that the periodicity in fibrin results from the orderly aggregation of preexistent structures in the manner of crystal growth. This concept of the mechanism is attractive in its simplicity, but cannot be supported by the existing evidence. It is not permissible to attribute this degree of significance to the *average* spacing without accounting specifically for the rather wide variation in spacing between separate fibrils. If the periodicity were constructed of such an orderly array, it should appear at all stages of aggregation, for example, in the finest fibrin fibrils. The nodose character of fibrinogen filaments is suggestive of a preexistent striation, but it lacks the extreme regularity of that in fibrin. Also, the electron microscope evidence indicates that there is a random distribution of filament lengths in fibrinogen.

It is noteworthy that, although there is considerable variation in spacing between separate fibrils, there is a high constancy in the spacing within individual fibrils or fibril bundles. It appears that there is an interdependence of spacing in contiguous structures and that, once initiated, the periodicity is propagated with considerable precision throughout the fibril. In future efforts to account for this anomalous distribution of matter, due attention should be given to the possible effects of colloidal forces in this essentially colloidal system. From the chemical standpoint the "molecular" units may very well be those particulate elements which border on the electron microscope limit of resolution, in the neighborhood of 50 A and less.

SUMMARY

1. The macro period in fibrin fibrils from bovine Fraction I is shown to consist of a narrow stain-receptive band mid-way between two denser and wider stain-receptive bands whose average distance center to center along the fibril axis is about 230 A.

2. Fibrin fibrils formed from a highly purified sample of human fibrinogen were shown to have a structure indistinguishable from that observed in the bovine preparations. The average dimension of the macro period was 215 A for the human material, which value is not considered to be significantly different from that of bovine fibrin, since there is a considerable variation in the value of the macro period between individual fibrils for both materials.

3. Within the range of resolution available for the investigation, stained fibrin fibrils appear to be constituted of particles having dimensions in the range of 30 to 50 A.

4. Bovine Fraction I and the sample of human fibrinogen were shown to consist in large part of filamentous elements with an average length of about 600 A and an estimated width of about 30 or 40 A. With the ultimate resolution available, fibrinogen filaments appear nodose, not unlike a string of beads.

5. It is concluded that fibrin is produced through a predominantly lateral association of fibrinogen filaments. The characteristic axial periodic structure is interpreted as consisting of periodic variations of protein concentration resulting from local axial displacements of material to preferred positions at an advanced stage of fiber formation. No significant relation was established between the lengths of fibrinogen filaments and the dimensions of the macro period in fibrin.

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EXPLANATION OF PLATE 3

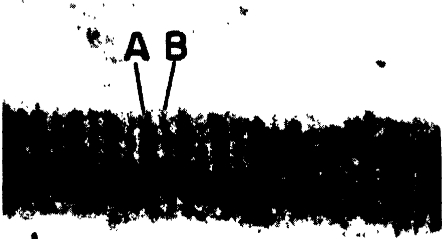
FIG. 1. Fibrin fibril from bovine Fraction I, clotted in 0.9 per cent NaCl at pH 6.5, stained with 0.1 per cent phospho-12-tungstic acid. × 217,000.

FIG. 2. Fibrin fibrils from purified human fibrinogen, clotted in 1 per cent NaCl, pH about 6, stained with 0.1 per cent phospho-12-tungstic acid. × 186,000.

FIG. 3. Bovine Fraction I, 0.01 mg. per cc. from 1 per cent NaCl, shadowed with uranium. × 164,000.

FIG. 4. (*a*) Collodion control film treated with distilled water. (*b*) Human fibrinogen, 0.05 mg. per cc. from 0.8 per cent NaCl. Shadow-cast with uranium. × 181,000.

FIG. 5. (*a*) Collodion control film. (*b*) Bovine Fraction I, shadowed with nickel. × 200,000.



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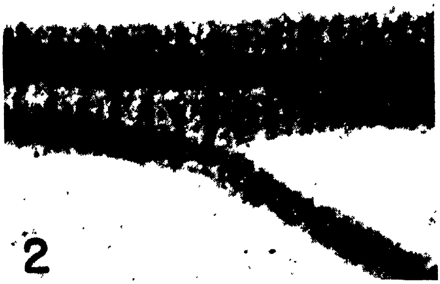

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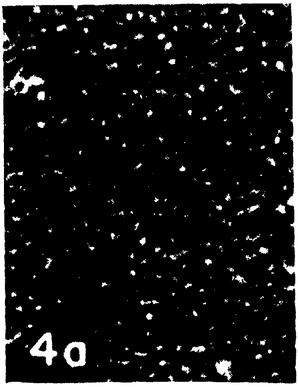
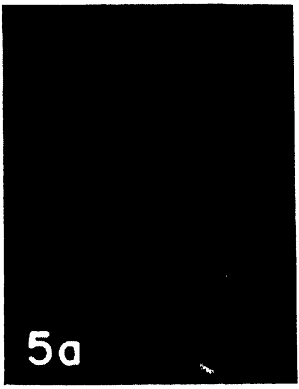


4a

b



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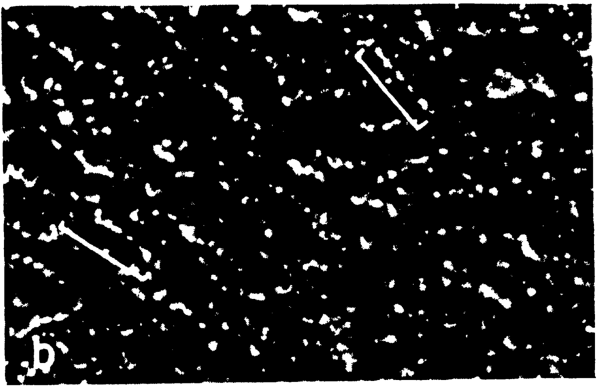

b

(Hall, Fibrinogen and fibrin)

A COLORIMETRIC METHOD FOR THE DETERMINATION OF PHENOL OXIDASE IN PLANT MATERIAL*

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Enzymes catalyzing the oxidation of phenols occur widely in plant tissues and have been extensively studied (1–7), but the diversity of their properties and the complexities of the reactions catalyzed have made assays and their interpretations uncertain. In only a few instances have essentially pure enzyme preparations been studied (1, 6, 8), and even in these cases the mechanism of oxidation is not well understood. The two principal types of phenol oxidase or tyrosinase can best be defined in terms of the substrates used for their detection; for example, catechol for catecholase or polyphenolase, and *p*-cresol or phenol for cresolase or monophenolase. In each case the first reaction product is believed to be a highly reactive *o*-quinone (1).

Early methods of determination (2, 5) were based on measuring the colored oxidation products of phenols or aromatic amines, but the conditions controlling color formation were complex with generally inaccurate results. More recently, manometric methods in which oxygen uptake is measured with catechol or *p*-cresol as substrates have been preferred. In the former case, in which the enzyme tends to be rapidly inactivated during the oxidation, a secondary substrate system has often been employed to keep the *o*-quinone reduced. Kubowitz (9) used the hexose monophosphate dehydrogenase-triphosphopyridine nucleotide system, while Nelson and Dawson (1) employed hydroquinone or ascorbic acid. This device, however, does not eliminate enzyme inactivation, since the latter appears to depend on the actual amount of oxygen reduced (1). This sets definite limits on the accuracy of manometric methods.

The goal of the present work was to provide a phenol oxidase method suitable for routine assay of large numbers of samples of plant material, as well as for the estimation of the enzyme in small amounts of tissue.

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A colorimetric method seemed to offer the greatest speed, simplicity, and sensitivity if certain difficulties of the earlier methods could be avoided. In the proposed method the oxidized phenolic substrate is continually reduced by a suitable leuco dye as follows:

$$\text{(a)} \qquad \text{Phenol} + O_2 \xrightarrow{\text{oxidase}} \text{quinone}$$

$$\text{(b)} \qquad \text{Quinone} + \text{reduced dye} \longrightarrow \text{phenol} + \text{oxidized dye}$$

Reaction (*b*) should be stoichiometric and rapid compared with reaction (*a*), so that the over-all reaction is limited by the enzymatic step. The reaction can then be followed photometrically by measuring the oxidized dye under conditions in which the quinone does not accumulate. If a dye with a high color intensity is chosen, only the very initial part of the reaction need be measured. Enzyme preparations from apple fruit and potato tuber were mainly used in the development of the method, but a variety of other plant materials were subsequently investigated.

EXPERIMENTAL

Conditions Affecting Colorimetric Reaction

The reactions were carried out in test-tubes at 30° with a phosphate-citrate (McIlvaine) buffer of pH 6.0. This pH was found to be close to the optimum for most of the enzyme preparations tested and was high enough to give the full blue color of the indophenol dye adopted without permitting appreciable autoxidation of the leuco dye. Variation in buffer strength had little effect on the rate of reaction. The temperature was adequately controlled by keeping all reagents in a 30° bath, since no significant change in temperature occurred during the very short reaction period required for the colorimetric determination. Rates were measured with the Lumetron colorimeter and multiple reflection galvanometer. Galvanometer readings plotted on a logarithmic axis were linear with time of the reaction for a suitable period under the conditions described.

Choice of Dye and Its Effect—A large number of reversibly oxidized dyes of suitable potential were tested from the standpoint of solubility, stability, autoxidizability, absorption coefficient, and effect on the enzyme preparations. The substituted indophenols were found most suitable and 2,6-dichlorobenzenoneindo-3′-chlorophenol (Eastman) was the best of these. It is readily reduced by hydrogen and palladized asbestos and is stable in the leuco form under hydrogen for several hours. It is conveniently standardized colorimetrically with ascorbic acid, but weighed samples of the dye gave sufficiently uniform solutions for routine work. For many purposes ascorbic acid reduction may also be acceptable for leuco dye preparation, but there was some evidence that the oxidation products of ascorbic acid affected the enzyme reaction adversely.

Two important effects of the indophenol dyes were observed that were further considered. First, the dyes varied widely in their effect on the oxidation rates of enzyme preparations from certain sources. With some dyes initial rates were low and fell off rapidly, indicating enzyme inhibition. The 3′-chloro dye was, in fact, originally substituted for the more common 2,6-dichlorobenzenoneindophenol, because the former gave higher rates of longer linear course with potato preparations. The potato enzyme was particularly sensitive to these dye effects, but was not typical of the plant materials investigated. A second important characteristic of the indophenol dyes in general and the 3′-chloro dye in particular was the fact that they were oxidized directly, without phenolic mediator, by enzyme preparations from some tissues. This will be referred to as "dye oxidase" activity[1] and will be discussed further in a later section. With such preparations, therefore, two measurements of oxidase activity were necessary, (*a*) total phenol oxidase (with phenolic substrate or mediator) and (*b*) "dye oxidase" (without phenolic substrate). The difference is considered a measure of true phenol oxidase activity. This is based upon two types of evidence. First, mixtures of two enzyme preparations of widely varying proportions of "dye oxidase" and phenol oxidase gave additive rates within experimental error, indicating no interaction of direct dye oxidation and dye oxidation through phenol mediation. Second, the two activities could be separated by a specific inhibitor as shown in a later section.

Fig. 1 illustrates the effect of the leuco dye[2] concentration on rate of the reaction with three representative types of enzyme preparations. It may be seen that apple oxidase had very little "dye oxidase" activity and was inhibited to some extent above a certain optimum dye concentration. Potato oxidase showed a large fraction of "dye oxidase" activity and marked inhibition by the dye. Carrot oxidase, on the other hand, showed a very high proportion of "dye oxidase," and no evidence of inhibition by the dye. The effect of two levels of dye concentration may also be noted incidentally with enzyme preparations from other sources in Table IV. It is clear that the effect of dye concentration varies from one tissue to another, and this fact must be considered in adopting a concentration for a standard technique.

Choice of Phenolic Substrate—A variety of phenolic compounds were tested for substrate or mediator activity in the system. Table I gives representative data for apple and potato enzyme preparations. Values greater than unity indicate mediator activity by the phenol, while values less than unity indicate inhibition of "dye oxidase" activity. It is evident that only catechol or its analogues were effective mediators. Chlorogenic and caffeic acids were actually somewhat more effective than catechol, while protocatechuic acid and dihydroxyphenylalanine were less effective. Of the monophenols, only tyramine and possibly tyrosine showed definite but small mediating effects, while other phenols, including *p*-cresol, were inactive. Catechol, therefore, was used in all subsequent work and the enzyme activity which it mediated is best termed *catechol oxidase.*

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[1] The term "dye oxidase" is used only as a temporary measure. Without further investigation of the nature and specificity of this enzyme, any more specific or permanent term seems unjustifiable at this time.

[2] Hereafter "dye" will indicate the 2,6-dichlorobenzenoneindo-3′-chlorophenol.

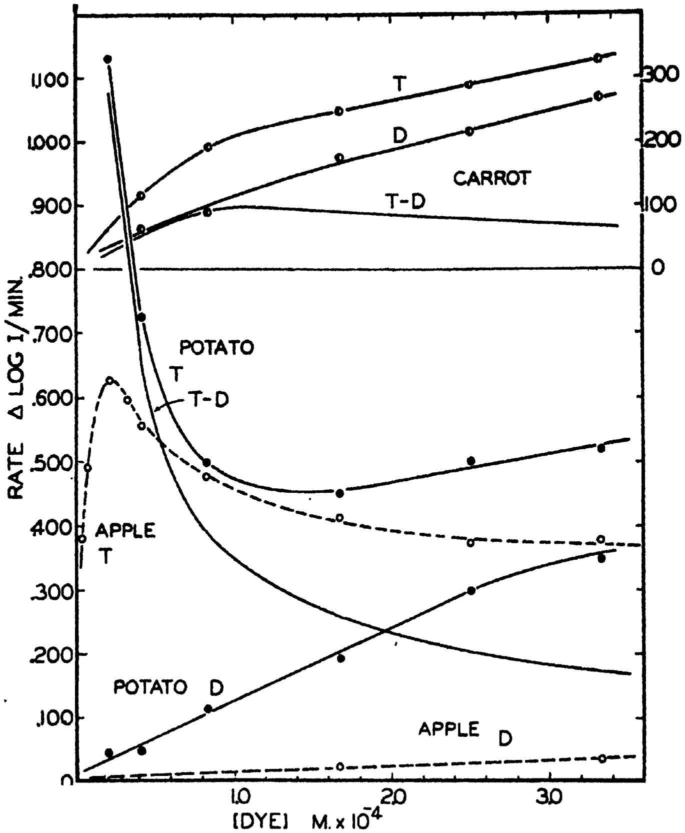

FIG. 1. The relation of leuco dye concentration to rate of oxidation by phenol oxidase of three typical plant enzyme preparations. T = total oxidase, D = "dye oxidase," and $T - D$ = catechol oxidase activity.

Fig. 2 demonstrates the effect of catechol concentration on rate of the reaction with apple and potato preparations. No true optimum concentration was reached and the higher concentrations were not favorable for use, since the individual rate curves fell off with time too rapidly for accurate measurement.

Fig. 3 illustrates the essential point that with suitable catechol and leuco dye concentrations the rates of reaction were directly proportional to enzyme concentration over at least a 10-fold range.

Enzyme Preparation

In routine assay of plant material it is desirable that preparation of the enzyme be rapid, simple, and adaptable to adequate sampling. Plant tissues, however, may contain interfering substances such as ascorbic acid and sulfhydryl compounds, phenol oxidation products with inhibitory effects on the enzyme, and acids producing an unfavorable pH in the homogenate. The following general procedure was designed to meet requirements and obviate the difficulties mentioned: (*a*) the tissue was ground with an excess of ascorbic acid at a suitable pH for enzyme stability, (*b*) crude proteins and cell fragments were precipitated at about −25° by 80 per cent acetone and centrifuged, and (*c*) the precipitate was resuspended in suitable buffer at 0°. Extensive experience with this procedure and several modifications showed, however, that the precautions

TABLE I

Effect of Various Phenols as Mediators in Oxidation of Leuco Dye

The figures reported represent the ratios of the rates found with added phenol (total oxidase) and without phenol ("dye oxidase"). Hence ratios greater than unity represent mediation by the phenol; less than unity, an inhibition of "dye oxidase."

Mediator	Potato enzyme			Apple enzyme			
	Concentration of phenol			Concentration of phenol			
	8.3×10^{-3} M	8.3×10^{-4} M	8.3×10^{-5} M	8.3×10^{-3} M	8.3×10^{-4} M	8.3×10^{-5} M	8.3×10^{-6} M
Catechol	1.89	1.30	1.09	34	5.5		
Protocatechuic acid	0.70	1.10	1.17	2.45			
Dihydroxyphenylalanine		1.10			2.22		
Caffeic acid		1.35			6.9		
Chlorogenic acid		1.92			9.1		
Pyrogallol		0	0.68		0	0.54	1.10
Gallic acid	0.17	0.89	1.00	0	0.91	0.98	
Tyrosine		0.76	0.92		1.05		
Tyramine	1.06			1.28			
Guaiacol	0.59	0.81	0.90	0.87	1.10		
Phenol	0.98			0.92			
Vanillic acid		0.88			0.76		
p-Methylaminophenol	0		0.58	0		0.98	

necessary in extraction and preservation of the enzymes varied considerably with the tissue and the method of sampling and grinding.

When adequate sampling required large amounts of tissue, 25 or 50 gm. were chopped in a Waring blendor and a small aliquot further ground in a Potter-Elvehjem homogenizer (10); with 1 gm. samples the latter homogenizer was used directly. Homogenization beyond the point of complete cell rupture did not increase activity, though in many preparations a large fraction of the activity was not in true solution and could be centrifuged at moderate speeds. The necessity for ascorbic acid addition during grinding varied. With potato tuber tissue it was clearly beneficial, but with other tissues, including apple, it was not required if homogenization was rapid and the preparation precipitated or assayed within a few minutes.

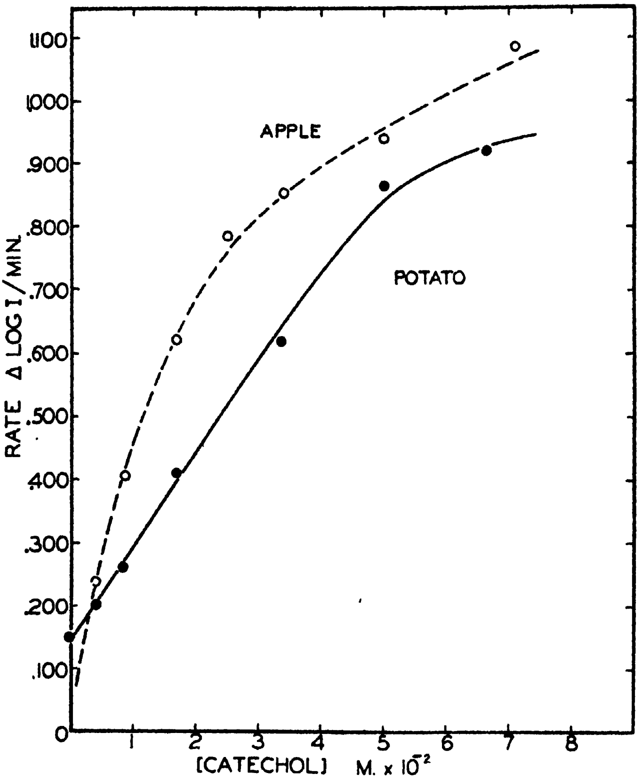

FIG. 2. The relation of catechol concentration to rate of dye oxidation with apple and potato enzyme preparations.

The pH during preparation of the enzyme had a marked effect with both apple and potato tissues. At values below pH 4 to 5, much lower activities were obtained than at higher values, as indicated for potato tuber tissue in Table II. In general, either homogenates or acetone

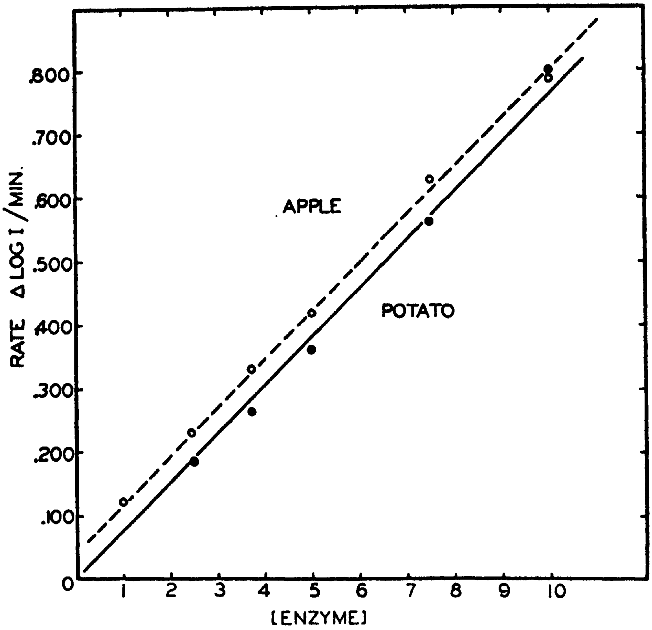

Fig. 3. The relation of enzyme concentration to rate of dye oxidation with apple and potato preparations.

Table II

Effect of pH on Stability of Oxidase Activities of Potato Homogenate

Aliquots of homogenate were suspended in buffers at the indicated pH values and incubated at 25° for the intervals indicated. Total oxidase activity and "dye oxidase" activity were determined, and catechol oxidase was calculated by difference. The data are given as per cent of the figures in bold-faced type.

Time	pH 7.5			pH 6.0			pH 4.5		
	Total oxidase	"Dye oxidase"	Catechol oxidase	Total oxidase	"Dye oxidase"	Catechol oxidase	Total oxidase	"Dye oxidase"	Catechol oxidase
min.									
0	**100**	**100**	**100**	95	90	99	54	24	79
15	94	99	91	98	93	103	44	20	65
45	91	87	96	79	73	85	40	10	66

preparations were sufficiently stable for assay purposes for 15 to 30 minutes at pH 6.0 even at room temperature.

The effect of acetone precipitation was also studied extensively with apple and potato tissue and to a lesser extent with several other tissues. The per cent acetone was not highly critical and 80 per cent was considered optimal. The effect of time and temperature indicated that subzero temperatures were not necessary for short periods (10 to 20 minutes) but advisable for longer exposure of the enzymes to acetone. When ascorbic acid addition was used, acetone precipitation was the most convenient method of removing the excess, although it could also be accomplished by aerating the homogenate as was practised in certain recovery studies. Acetone precipitates were generally more stable than homogenates and in some cases gave better rate curves. Acetone precipitation also prevented darkening of preparations such as apple and potato by removing most of the natural substrates. Recoveries on acetone precipitation, as seen in Table III, were not uniformly good; potato was most consistent, apple more variable, and some other tissues showed only poor recovery.

TABLE III

Recovery of Oxidase Activities after Acetone Precipitation with Several Plant Tissues

Data given as per cent recovery in acetone precipitate based on activity of the homogenate with ascorbic acid removed, when added, by aeration.

Tissue and conditions	Total oxidase	"Dye oxidase"	Catechol oxidase
Potato, pH 6.0, with AA[*]	84–104 (97)[†]	52–64 (61)[†]	102–132 (114)[†]
" " 6.0, without AA	60– 85 (75)[†]	37–54 (48)[†]	75– 96 (85)[†]
Apple, pH 6.5, with AA	89–135 (107)[†]		
Sweet potato, pH 7.6, with AA	79	86	76
" " " 7.6, without AA	66	58	72
Mushroom, pH 7.7, with AA	71		
" " 7.7, without AA	66		

[*] AA = ascorbic acid.

[†] Range and average of six separate trials. The figures in parentheses represent the averages.

It was found that for most tissues investigated, when ascorbic acid addition was not essential and when high dilution was possible, acetone precipitation was not required to produce enzyme preparations sufficiently stable and free of interfering materials for the assay. Over-all dilutions for the preparations illustrated in Table IV, for example, ranged from 1:500 for carrot and 1:2000 for apple and potato to 1:25,000 for mushroom.

Standard Procedure

Reagents—

Leuco dye. The commercial dye (Eastman) was used without further purification, but each lot was standardized colorimetrically with ascorbic acid. 0.001 M dye solutions were prepared as follows: The calculated amount of solid (35 mg. in the case of the lots used) is dissolved in 100 ml. of distilled water by shaking periodically for 20 to 30 minutes, and then filtering off the undissolved material. This solution is stable for 3 or 4 days at room temperature.

TABLE IV

Phenol Oxidase Activities of Various Plant Tissues Measured Colorimetrically and Manometrically

Activities computed for both methods as micromoles × $ml.^{-1}$ × $min.^{-1}$ × $gm.^{-1}$ (fresh weight), where 1 mole of dye is equivalent to 1 gm. atom of oxygen.

Tissue	Leuco dye added	Colorimetric method				Manometric method		
		Total oxidase	"Dye oxidase"	Catechol oxidase	Catechol oxidase / "Dye oxidase"	Catechol oxidase	Cresolase	Catechol oxidase / Cresolase
	µM							
Potato (tuber)	0.5	16.2	3.2	13.0	4	17.8	7.1	2.5
	2.5	22.7	15.9	6.8	0.4			
Apple (fruit)	0.5	14.8	0.102	14.7	144	11*	0.3	36
	2.5	8.9	0.32	8.6	27			
Sweet potato (tuber)	0.5	14.1	4.1	10.0	2.4	2.9*	0	
	2.5	26.4	15.8	10.6	0.7			
Beet (root)	0.5	74.8	6.8	68.0	10	10.9	12.2	0.9
	2.5	44.8	22.0	22.8	1			
Peach (fruit)	0.5	12.9	2.14	10.8	5	4.0	0.21	19
	2.5	12.5	4.7	7.8	1.7			
Banana (fruit)	0.5	23.5	0.63	22.9	36	18.5	4.0	4.6
	2.5	28.0	2.53	25.5	10			
Asparagus (stem)	0.5	11.0	3.4	7.6	2.2	0	0	
	2.5	22.7	15.2	7.5	0.5			
Mushroom	0.5	514	0.35	514	1470	148	23	6.5
	2.5	360	0.38	360	950			
Carrot (root)	0.5	5.3	2.57	2.7	0	0		
	2.5	13.3	12.0	1.3	0.11			

* Estimated initial values from rapidly declining rate curves.

To prepare the leuco dye for a single day's use, an appropriate amount of the above stock is diluted 5-fold (0.0002 M) and about 1 ml. of a freshly prepared 0.2 per cent suspension of 5 per cent palladized asbestos is added to each 200 ml. of dilute dye. Hydrogen is then bubbled through the solution just to the point of complete reduction, 3 to 4 ml. of 0.1 M phosphate-citrate buffer (pH 6.0) are added to reduce autoxidizability, and the solution filtered quickly through retentive paper on a Büchner funnel. A slow stream of hydrogen passing through the solution is maintained for the period of use of the leuco dye. The 5 per cent palladized asbestos

(Fisher Scientific Company) should be washed thoroughly with distilled water and oven-dried. Normally the reduction occurs within 15 minutes and the filtered solution is colorless. If the filtrate is appreciably red, either too much palladized asbestos was used or the hydrogen bubbling was continued too long after complete reduction.

Catechol solution. Freshly prepared 0.1 M catechol containing a drop of 10 per cent acetic acid per 100 ml.

Method

The following procedure was found suitable for most plant materials. A weighed sample of tissue, fresh or frozen, is ground with water in a Waring blendor and homogenizer, or homogenizer alone, depending on the sample size, until the cells are ruptured. Sufficient 0.2 M K_2HPO_4, varying with the acidity of the tissue, is included to maintain a pH of about 7.0. Dilution of the tissue in the homogenate is 1:10. The homogenate is then diluted to the proper concentration for satisfactory rate measurement and assayed as soon as possible. When delay is unavoidable, the preparation should be kept in an ice bath or, if found suitable for the particular tissue, an acetone precipitate may be prepared.

To a colorimeter tube (20 × 150 mm. test-tube) are added 5.0 ml. of 0.1 M phosphate-citrate (McIlvaine) buffer of pH 6.0, 5.0 ml. of 0.0002 M leuco dye (1.0 μM), 1.0 ml. of 0.1 M catechol, and finally 1.0 ml. of enzyme suspension, all reagents at 30°. The tube is quickly swirled for mixing and inserted in the colorimeter (645 mμ filter), and the lamp rheostat adjusted to give a suitably high (80 to 100 per cent) transmission. Galvanometer readings are then taken at 5 second intervals, usually during the 15 to 45 seconds after mixing. A timer with a flashing light signal is convenient when working alone.

The per cent transmission (I) plotted against time on semilog paper normally gives a linear relation for at least 30 to 60 seconds, and the slope of the line is a convenient measure of the reaction rate. When rates are known to be linear, two readings may be sufficient and plotting unnecessary. Since the blank transmission (I_0) does not change significantly during the reaction, the rate of increase in optical density (D) is measured by the slope and, by Beer's law, the rate of increase in oxidized dye concentration (C). For a dye of absorption coefficient ϵ and standard tube of light path L, the rates may be expressed as change in log I or D per minute as follows:

$$\text{(1)} \qquad \text{Initial reading, } \log I_0 - \log I_1 = \epsilon \times L \times C_1 = D_1$$

$$\text{(2)} \qquad \text{Final reading, } \log I_0 - \log I_2 = \epsilon \times L \times C_2 = D_2$$

Subtracting equation (1) from equation (2) gives

$$-(\log I_2 - \log I_1) = \epsilon \times L \times (C_2 - C_1) = D_2 - D_1$$

or

$$-\Delta \log I = \epsilon \times L \times \Delta C = \Delta D$$

$$\text{Rate} = \frac{\Delta \log I}{\Delta T} = \epsilon \times L \times \frac{\Delta C}{\Delta T} = \frac{\Delta D}{\Delta T}$$

Such rates were converted to an absolute basis for the particular instrument, dye, and tube used as follows:

$$\frac{\Delta \log I \text{ per min.} \times \text{dilution factor}}{3.50 \times \text{volume}} = \frac{\text{micromoles}}{\text{ml.} \times \text{min.} \times \text{gm.}}$$

where "dilution factor" is the volume of enzyme preparation divided by the fresh weight of tissue, "volume" is that of the reaction mixture, usually 12 ml., and 3.50 is the optical density of 1 μM of oxidized dye in a 12 ml. volume under the conditions used.

Some Applications and Properties of Method

Quantitative comparison of phenol oxidase activities by different methods is always difficult and especially so with crude catechol oxidase preparations which may be inactivated during their action. Since manometric methods have been most common, a series of comparisons of the colorimetric and manometric methods was made with enzyme preparations from a variety of plant tissues. The manometric technique was similar to that of previous workers (1) except that the pH, buffer strength, and catechol concentration were adjusted slightly to those of the colorimetric method.

Several points of comparison are illustrated in Table IV. It is significant that accurate colorimetric measurements could be made in cases in which the manometric method failed, giving poor rates or none at all. This may have been due in some cases to the longer time necessary to make the manometric observations, but in other cases, for example carrot, it seemed to be due to a reversible inactivation of the enzyme, since the contents of the Warburg flask showed a large fraction of the original activity by subsequent colorimetric measurement. The catechol oxidase activity by the colorimetric method, especially at dye concentrations at which inhibition was minimum, was always equal to that measured by the manometric method, and usually much greater. This is consistent with the view of Nelson and Dawson (1) that the amount of inactivation of the enzyme is proportional to the oxygen uptake, which is necessarily much greater in the manometric method.

The sensitivities of the two methods can be compared on the basis of the relative oxygen uptakes required to give minimum values of comparable precision in the instruments used in the respective methods. On this basis an oxygen uptake of 25 μl. per hour in the Warburg apparatus is 300 times that equivalent to a Δ log I per minute of 100 in the colorimetric method.

Table IV further shows the wide range observed in the total oxidase activity, in the proportion of "dye oxidase" and catechol oxidase, and in behavior with respect to dye concentration. Carrot oxidase, for example, was 90 to 95 per cent direct "dye oxidase" at the higher dye concentration, while mushroom oxidase was never more than 0.1 per cent "dye oxidase."

"Dye Oxidase"

Since peroxidase and cytochrome oxidase (with appropriate substrates) also catalyze leuco dye oxidation, their possible relation to "dye oxidase" activity was examined. Peroxidase would require the presence of a suitable concentration of peroxide in the enzyme preparation. The presence of an adequate concentration of peroxide seemed improbable, but this possibility was eliminated by the response of oxidase preparations to added purified peroxidase and catalase. The presence of cytochrome oxidase was also rendered unlikely, first, since some of the preparations were essentially soluble in contrast to typical cytochrome oxidase preparations, and second, because addition of cytochrome *c* failed to catalyze dye oxidation which it does in the presence of heart muscle or wheat germ preparations known to contain cytochrome oxidase.

There was the further possibility that "dye oxidase" was related to other types of phenol oxidases. In a sense the leuco dye is a *p*-aminophenol toward which laccases are generally active; however, the "dye oxidase" preparations did not oxidize hydroquinone or *p*-phenylenediamine, both of which are typical substrates for laccase.

Another possible cause of direct oxidation of the dye might be monophenolase or cresolase activity which is known to occur in many of the tissues studies (1, 3). Though unlikely on several grounds, this was a possibility more difficult to exclude. However, *p*-cresol and other monophenols, with the possible exception of tyramine, were shown not to mediate dye oxidation. Also, no clear correlation was found between "dye oxidase" and manometric cresolase activities in a variety of plant tissues. This may be seen in Table IV in which "dye oxidase" determined by the colorimetric method and cresolase activity by the manometric method were compared, as well as the ratios of catechol oxidase to "dye oxidase" and catechol oxidase to cresolase. This comparison cannot be exact, but there seems to be no consistent tendency to indicate the identity of "dye oxidase" and cresolase. The "dye oxidase" was also differentiated from cresolase by the use of the inhibitor phenylthiocarbamide. As shown in Table V, there is little difference in the sensitivities of catechol oxidase and cresolase to this inhibitor as determined manometrically, while catechol oxidase and "dye oxidase" show markedly different inhibitions to phenylthiocarbamide.

Finally there is the possibility that the "dye oxidase" activity is not actually a direct one but that some natural mediator is present in the preparation. If such were the case, however, it is not removed by acetone precipitation, nor is it liberated upon heat coagulation of the protein, since concentrates of the supernatant fluid after heat denaturation do not activate catechol oxidase preparations. The additive properties of "dye oxidase" and catechol oxidase activities also argue against the functioning of a mediator in "dye oxidase" activity. It can only be concluded at present that "dye oxidase" is probably some type of phenol oxidase, not typical of laccase or cresolase, which is especially active toward indophenols.

TABLE V

Per Cent Inhibition of Phenol Oxidases by Phenylthiocarbamide

Tissue	Method	Oxidase activity	Concentration of phenylthiocarbamide					
			10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M	8×10^{-3} M
Apple	Colorimetric	Catechol oxidase				44	76	100
Potato	"	" "				7	71	100
	"	Dye oxidase				2	0	16
	Manometric	Catechol oxidase	0	74	88			
	"	Cresolase	9	56	96			
Beet	Colorimetric	Catechol oxidase		2	27	84	95	
	"	Dye oxidase		0	0	0	0	
	Manometric	Catechol oxidase	27	58	100			
	"	Cresolase	5	30	85			

Micro Modification of Method

The colorimetric method offers the possibility of measuring phenol oxidase in minute quantities of tissue for physiological investigation. The high absorption coefficient of the oxidized dye at 645 mμ affords high sensitivity even in the procedure described, but this can easily be increased 100-fold by reduction in volume and increase in reaction time. The following procedure was developed with apple fruit tissues. Microtome slices of frozen tissue weighing 0.1 to 0.5 mg. were homogenized in a small Potter-Elvehjem type homogenizer (approximately 5 × 25 mm.) containing 100 μl. of buffer and excess ascorbic acid. The homogenate was transferred with a micro pipette to a small conical centrifuge tube (7 × 35 mm.) with another 100 μl. of buffer for rinsing. The enzymes were precipitated with 800 μl. of acetone at −25° and resuspended in 100 μl. of buffer of pH 6.0. Rate determinations of from 1 to 3 minutes duration were made with Lowry and Bessey's adaptation of the Beckman spectrophotometer (11) with 50 μl. constriction pipettes to transfer suitable amounts of catechol, leuco dye, and enzyme to micro cuvettes (2 × 10 mm. inner dimensions). Results were of nearly as high precision as in the larger scale procedure, and the sensitivity, equivalent to a minimum oxygen uptake of approximately 0.0002 μl., is comparable to those of the histochemical methods of Linderstrøm-Lang and Holter (12).

DISCUSSION

The colorimetric method described has several advantages in speed, convenience, and sensitivity over previous methods of measuring phenol oxidase activity. Complete determinations can be made in as little as 5 to 10 minutes and a team of two workers can carry out ten per hour with most plant materials. The method is also readily adapted to small scale techniques for determinations on a histochemical level.

The use of a leuco dye as a secondary substrate, however, has introduced some complexities. The variable inhibitory effect of the indophenol dye on oxidase from certain plant materials necessitates control of dye concentration and restricts the comparison of enzyme activities from different sources. The latter is difficult, however, by any method, since phenol oxidase activity is so generally dependent on the substrate and experimental conditions. It may also be pointed out that the dye used in this method gave much higher sensitivity (at least 10 times) and better rate curves than the pyrogallol, benzidine, or nadi reagent used in earlier colorimetric methods.

The occurrence of direct "dye oxidase" activity in some plant preparations was a further complication, but it appears different from catechol-mediated dye oxidation and may in itself prove to be an interesting part of the phenol oxidase mechanism of plant tissues. The identification of "dye oxidase" will require further work.

The almost complete lack of activity of monophenols as mediators seemed to rule out the use of the method for monophenolase determination. The reason is uncertain, since *o*-quinones are considered to be formed in the enzymatic oxidation of monophenols as well as *o*-dihydroxyphenols (1).

Considerable variation was found in the properties of enzyme preparations from different plant materials which called for adjustment in the method of preparation in some cases. However, a general procedure has been presented which was adequate for the majority of tissues investigated. It is characterized by thorough cell rupture, avoidance of low pH, short standing, and high dilution which usually obviated further purification of the homogenate.

The same general technique has been adapted to the determination of peroxidase in plant material (13) and much of the work was concurrent with that in the present report. The method has also been adapted for cytochrome oxidase determination (14) in which cytochrome *c* replaces catechol as the primary substrate or mediator. Thus it appears feasible to determine three important respiratory oxidases by a single method of improved speed, convenience, and sensitivity.

SUMMARY

A new colorimetric method for determining phenol oxidase activity in plant tissues is described. It affords greater speed and sensitivity, being adaptable both to routine assay and to microanalysis on a histochemical level.

The enzymatically oxidized phenolic substrate, catechol, is continually reduced by leuco-2,6-dichlorobenzenoneindo-3′-chlorophenol, and the rate of color formation, measured photometrically over a short period, is found to be linear and directly proportional to enzyme concentration under suitable conditions.

The influence of leuco dye and catechol concentration and of the method of enzyme preparation with various plant materials was investigated and a standard procedure suitable for most materials is presented.

Some preparations showed direct "dye oxidase" activity; that is, they catalyzed the oxidation of leuco dye without added mediator. This enzyme appeared to be distinct from catechol oxidase, cresolase, cytochrome oxidase, or peroxidase.

Comparison of the present colorimetric method with a conventional manometric technique was made on a group of representative plant tissues.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF PEROXIDASE IN PLANT MATERIAL*

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In a previous paper (1) a colorimetric phenol oxidase method was presented which utilizes catechol as a mediator and in which the oxidation of a leucoindophenol is followed photometrically over a very short time interval. During the development of this method it was found that the same basic method and technique were applicable to the determination of peroxidase. In the latter case, oxidation of the leucoindophenol dye is catalyzed directly by peroxidase in the presence of hydrogen peroxide. Since it is often desirable to determine both phenol oxidase and peroxidase in a given plant material, a basically similar method for both enzymes was considered advantageous.

Most previous peroxidase methods have also been colorimetric and have utilized a variety of oxygen acceptors such as pyrogallol (2–4), leucomalachite green (5, 6), guaiacol (6), nadi reagent (7), and others. Lucas and Bailey (8) have also used an indophenol dye similar to that in the present work, but in a quite different way. Another method commonly used, especially in the assay of plant material, is that of Balls and Hale (9), in which pyrogallol is the oxygen acceptor, but the utilization of hydrogen peroxide is measured.

This paper includes (*a*) a brief discussion of factors involved in the proposed colorimetric reaction and in the enzyme preparation, (*b*) a description of the method adopted, (*c*) some results of its application to plant materials, and (*d*) some points of comparison with other methods.

EXPERIMENTAL

Colorimetric Reaction

The same general technique as that of the phenol oxidase method (1) was found suitable for peroxidase determination. A pH of 6.0 for the colorimetric reaction proved optimal for enzyme preparations from various sources, and at the same time permitted only a negligible rate of oxidation of the leuco dye by peroxide in the absence of enzyme. Buffer strength was not critical. Much less variation of peroxidase activity with different indophenol dyes was encountered than in the case of phenol oxidase. Although the common 2,6-dichlorobenzenoneindophenol was quite satisfactory, 2,6-dichlorobenzenoneindo-3′-chlorophenol afforded somewhat greater sensitivity, as shown in Table I. Since the latter dye had been adopted for the phenol oxidase method, some convenience as well as sensitivity was gained by also selecting this dye for the peroxidase determination. No evidence of enzyme inhibition by the dyes was observed, and small differences in the sensitivity of the method with the various dyes were due essentially to variations in their absorption coefficients.

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TABLE I

Activities and Properties of Oxygen Acceptors in Peroxidase Reaction

Enzyme preparation	Acceptor*	Concentration	H_2O_2†	Rate‡	Michaelis constant	ε§
		M	*per cent*	*per cent*		
Apple (acetone ppt.)	3467	3.4×10^{-4}	0.04	100	1500	25
Same	3463	3.4×10^{-4}	0.04	60		15
"	Pyrogallol	0.033	0.04	1.1	1700	2.8‖
"	Leucomalachite green	1.2×10^{-4} *ca.*	0.0004	0.3	6	50
Potato (acetone ppt.)	3467	3.4×10^{-4}	0.04	100		
Same	3463	3.4×10^{-4}	0.04	61		
Horseradish (partially purified)	3467	3.4×10^{-4}	0.04	100		
Same	3463	3.4×10^{-4}	0.04	55		

* 3467 (Eastman Kodak Company), 2,6-dichlorobenzenoneindo-3′-chlorophenol; 3463 (Eastman Kodak Company), 2,6-dichlorobenzenoneindophenol; pyrogallol, 410 mμ filter used in measurement; leucomalachite green, in pH 4.0 buffer, 615 mμ filter used.

† All H_2O_2 concentrations were optimal.

‡ Rates for each enzyme preparation relative to those in bold-faced type.

§ ϵ = absorption coefficient = $(\log I_0/I)/(L(\text{cm.}) \times C$ (mM per liter)).

‖ This value is for purpurogallin in alcohol.

The effects of leuco dye and peroxide concentrations on the rate of the enzyme reaction were necessarily interrelated. These relations are illustrated for an apple preparation in Figs. 1 and 2. It will be seen that the optimal peroxide concentration in the reaction mixture was at about 0.04 per cent for a leuco dye concentration of 3.5×10^{-4} M. At this peroxide concentration the leuco dye was not at an optimal concentration. With lower peroxide concentrations an optimal dye concentration could be reached, but this was at such a level that there was danger of precipitation of dye in the stock solutions. Thus the best practical choice for maximum sensitivity seemed to be about 0.04 per cent peroxide and 3 to 4 × 10^{-4} M leuco dye. Under these conditions good proportionality between rate of oxidation of the leuco dye and enzyme concentration was obtained, as illustrated in Fig. 3.

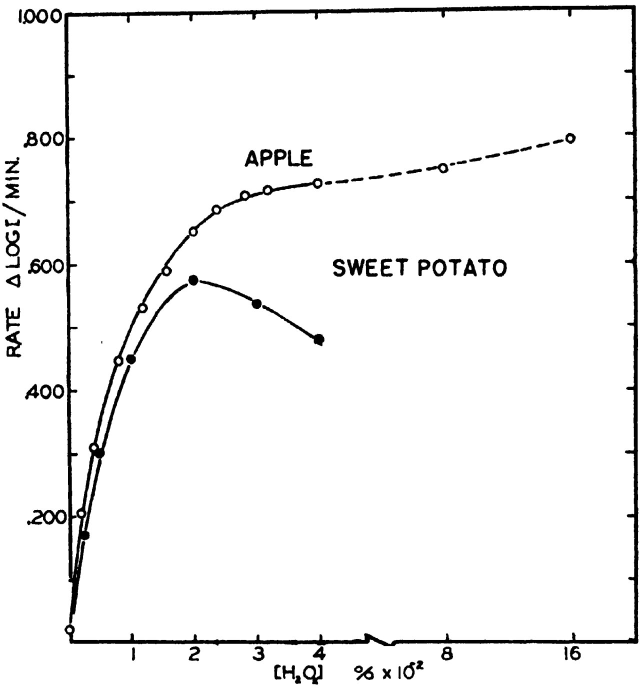

Fig. 1. The effect of peroxide concentration on the peroxidase reaction with apple and sweet potato preparations. Leuco dye concentration, 3.5 × 10^{-4} M.

It may be pointed out that enzyme preparations vary in their sensitivity to peroxide, as previously reported (10). As may be noted in Fig. 1, the sweet potato preparation was markedly inhibited by peroxide concentrations above the optimum, while an apple preparation showed little or no inhibition at such levels. This variation in sensitivity was considered in the design of a routine method. Inhibitory peroxide concentrations were usually indicated by non-linear rate curves and were generally avoided.

Enzyme Preparation

Much of the present work was carried out simultaneously with studies on the phenol oxidase method (1) and included investigation of the effects of sampling and grinding techniques, pH, ascorbic acid addition, acetone precipitation, temperature, and time. Peroxidase was similar in its behavior to phenol oxidase in many respects but showed generally greater stability. A pH of 7 to 8 was found to be optimal for the stability of peroxidase during preparation for apple and potato tissue. There was no evidence that the use of ascorbic acid during grinding was beneficial. Recoveries of enzyme activity after 80 per cent acetone precipitation were usually better than 90 per cent, but this procedure was not required, since simple homogenates were found to be as stable as acetone precipitates. Peroxidase differed from phenol oxidase in that diluted homogenates were more stable in peroxidase activity in the presence of gelatin. It was generally possible to use the same enzyme preparation for the determination of both peroxidase and phenol oxidase activities, except that the aliquot of homogenate for peroxidase determination was diluted with gelatin solution.

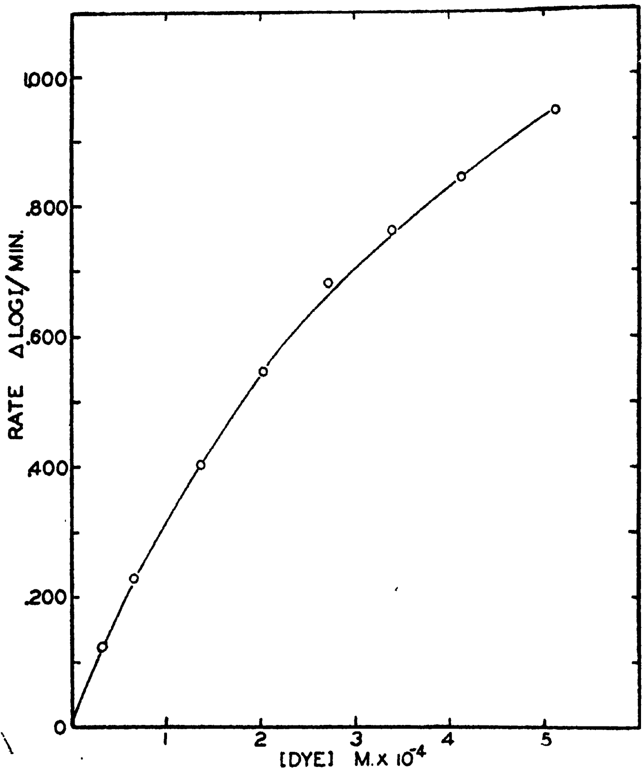

Fig. 2. The relation of leuco dye concentration and rate of the peroxidase reaction with an apple preparation. Peroxide concentration, 0.04 per cent.

Standard Procedure

Reagents—

Leuco dye. An approximately 0.001 M solution of 2,6-dichlorobenzenone-indo-3'-chlorophenol (35 mg. per 100 ml.) is prepared. To obtain the leuco dye reagent, the above stock is first diluted, after ascorbic acid standardization, to 5.5 × 10^{-4} M. The dye is reduced by first adding 2 ml. of a freshly prepared 0.2 per cent suspension of 5 per cent palladized asbestos for each 200 ml. of solution and then bubbling hydrogen through the solution until colorless. 3 to 4 ml. of 0.1 M phosphate-citrate buffer (pH 6.0) per 200 ml. of solution are added to reduce autoxidizability, the solution is quickly filtered through retentive paper in a Büchner funnel,

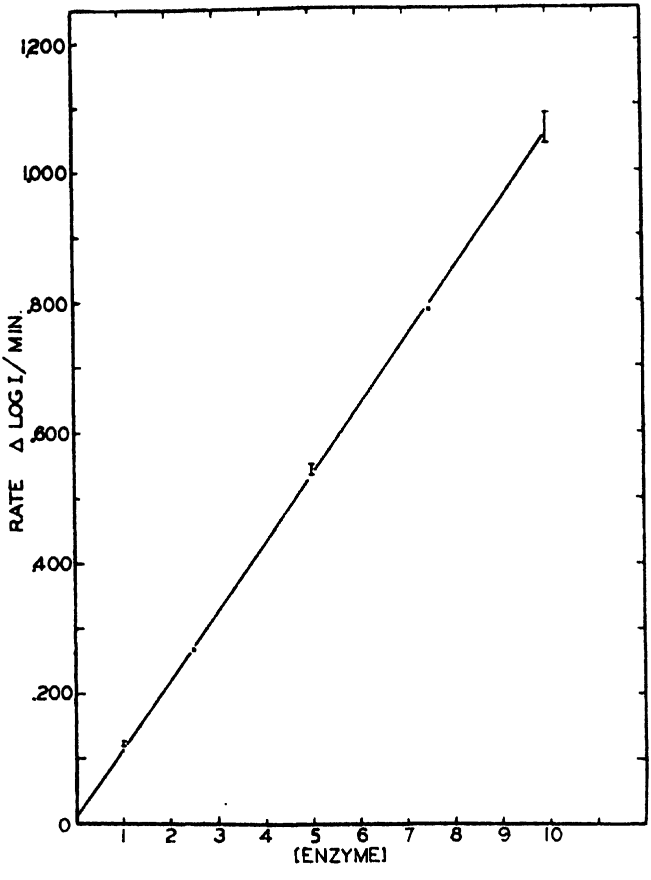

FIG. 3. The relation of enzyme concentration and rate of the peroxidase reaction with an apple preparation. Peroxide concentration 0.04 per cent, leuco dye concentration 3.5 × 10^{-4} M. The bars represent the range of five replicate determinations.

and the hydrogen bubbling is resumed during the use of the leuco dye reagent. Further detail on the leuco dye preparation is given in the description of the phenol oxidase method (1).

Hydrogen peroxide. 0.48 per cent hydrogen peroxide is prepared fresh from 30 per cent "superoxol."

Method

The following procedure was adopted for routine determination in plant materials: (1) A weighed sample of tissue, fresh or frozen, is ground first in a Waring blendor and then a portion in the Potter-Elvehjem homogenizer (11), or in the homogenizer alone if the sample is small, until the cells are ruptured. Sufficient 0.2 M K_2HPO_4, depending on the acidity of the tissue, is included to maintain a pH of 7 to 8. Dilution of tissue in the homogenate is 1:10. The homogenate is diluted to the appropriate concentration for assay with 0.5 per cent gelatin solution and assayed as soon as possible or stored for short periods in an ice bath. (2) To a colorimeter tube (20 × 150 mm. test-tube) are added 2.5 ml. of 0.2 M phosphate-citrate buffer (pH 6.0), 7.5 ml. of 5.5×10^{-4} M leuco dye, 1.0 ml. of 0.48 per cent hydrogen peroxide, and finally 1.0 ml. of the enzyme preparation. The tube is quickly swirled for mixing and inserted in the colorimeter with a 645 mμ filter. After adjusting the lamp rheostat to give a transmission of 80 to 100 per cent, galvanometer readings are taken at 5 second intervals, usually in the interval from 15 to 45 seconds after mixing. An interval timer with a flashing light signal is convenient.

Transmission values are plotted against time on semilog paper, and the rate was determined from the straight line and expressed as Δ log *I* per minute. These relative rates can be converted to an absolute basis by the following expression:

$$\frac{\Delta \log I \text{ per min.} \times \text{dilution factor}}{3.50 \times \text{volume}} = \text{rate}\left(\frac{\text{micromoles}}{\text{ml.} \times \text{min.} \times \text{gm.}}\right)$$

where "dilution factor" is the volume of enzyme preparation divided by the fresh weight of tissue, "volume" that of the reaction mixture, usually 12 ml., and 3.50 is the optical density at 12 ml. volume in the standard tube of 1 μM of oxidized dye. A more detailed explanation of the expression "Δ log *I* per minute" as a measure of rate is given in the report on phenol oxidase (1).

Some Applications and Properties of Method

Possible interference in the proposed method by other oxidative enzymes was evaluated. Direct oxidation of the leuco dye (without peroxide) occurred in some plant tissues, the so called "dye oxidase" reaction previously described (1). The extent of this reaction may be noted in Table II. It is conceivable that phenol oxidase and cytochrome oxidase in the presence of their natural substrates could interfere, though this is unlikely because of the high dilution of the tissue. Catalase might interfere by competing for the peroxide substrate. Studies with varying proportions of peroxidase and catalase[1] showed that relatively high catalase concentrations were necessary for appreciable interference which was evidenced by falling off in the rate curves. No such interference was encountered in the plant materials investigated.

TABLE II

Peroxidase Activity of Various Plant Tissues

Tissue	Dilution	Activity, μM × min.$^{-1}$ × ml.$^{-1}$ × gm.$^{-1}$, fresh weight		
		Total, with H_2O_2	Control, without H_2O_2	Peroxidase (difference)
Apple fruit, fresh	1:400	120	7.3	113
Potato tuber, fresh	1:1,000	362	68	294
Horseradish root, frozen	1:30,000	7030	0	7030
Carrot root, fresh	1:200	63	22	41
Beet root, frozen	1:2,000	423	65	358
Sweet potato tuber, frozen	1:2,000	490	78	412
Peas, frozen	1:1,000	128	27	101
Spinach, frozen	1:1,000	153	17	136
Mushroom, frozen	1:10	1.09	0.44	0.65
Cabbage, fresh	1:5,000	1905	0	1905
Asparagus stems, fresh	1:2,000	336	33	303
Radish roots, fresh	1:2,000	423	0	423
Celery tops	1:100	31	2.9	28

Table I shows that the leuco dye used in the present method affords much greater sensitivity of measurement than either pyrogallol or leucomalachite green. With pyrogallol, with which the Michaelis constant was found to be similar to that when the indophenol dye served as acceptor, the difference seemed to be due to the relative absorption coefficients and possibly to some inhibitory effect of pyrogallol on the enzyme. With malachite green, however, which has a higher absorption coefficient than the indophenol dye, the difference in sensitivity would seem to result from differences in the specific reaction rates of the intermediate compound, peroxidase-H_2O_2, with the two oxygen acceptors (see Chance (5)). Guaiacol and nadi reagent were found to be much less satisfactory in the present method than any of the above acceptors. The high sensitivity of the method was also demonstrated by comparison with the Balls-Hale method (9) in blanching studies[2] on peas and beans. Accurate determinations of fractional per cents of residual peroxidase activity after blanching were possible only with the present method.

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[1] We are indebted to Professor J. B. Sumner of the Biochemistry Department of Cornell University for a sample of crystalline beef liver catalase.

Much of the work reported in this paper was done with apple, potato, and horseradish preparations, but a survey of other available plant materials was made to test the method further. The results in Table II show the wide range of peroxidase activity encountered and the magnitude of the control rate (without peroxide). Most of this may be due to "dye oxidase," but since the preparations were all unpurified homogenates, some may have been due to other oxidases with their natural substrates, at least when the homogenate could not be greatly diluted.

DISCUSSION

The indophenol method has been readily adapted for peroxidase determination with the same technical advantages of speed, convenience, and sensitivity as that described previously for the phenol oxidase method (1). The leuco dye selected afforded higher sensitivity than either the pyrogallol or leucomalachite green acceptors which have been most commonly used in the past. The method has one disadvantage in comparison with the Balls-Hale method (9) that applies, however, to any method which employs an acceptor that may be an oxidase substrate and which measures substrate change. With materials of relatively low peroxidase activity and high "dye oxidase" activity, control determinations without peroxide are necessary and peroxidase activity must be calculated by difference.

As has been observed with pyrogallol and malachite green (6, 10, 12), excessive peroxide concentrations may cause suboptimal rates with some enzyme preparations. Although this was only rarely observed in the present work, it may complicate the use of fixed peroxide concentrations for the assay of a wide variety of tissues. A single peroxide concentration, therefore, may not serve to compare the peroxidase activities of different tissues accurately, but would be valid for comparing changes in activity in the same or similar tissues.

Preparation of the tissue for peroxidase analysis was similar to that for phenol oxidase (1); hence with most materials the same preparation served for both determinations.

SUMMARY

A new method of increased speed and sensitivity for determining peroxidase in plant tissue is described. Leuco-2,6-dichlorobenzenoneindo-

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[2] We are indebted to Dr. Z. I. Kertesz and his coworkers for the Balls-Hale determinations.

3′-chlorophenol is oxidized by peroxidase in the presence of hydrogen peroxide, and the rate of color formation is found to be linear and proportional to enzyme concentration under the conditions described. The influence of peroxide and leuco dye concentration and of the method of enzyme preparation was investigated and a standard procedure adopted. Assay results from representative plant tissues illustrate the flexibility of the method and comparative data with other peroxidase methods emphasize its sensitivity.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF CYTOCHROME OXIDASE*

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The colorimetric determination of phenol oxidase (1) and peroxidase (2) in plant tissues has been adapted for the determination of cytochrome oxidase in animal tissues. Previous methods for the latter have been manometric and it was thought that with so labile an enzyme the speed of the colorimetric method might be advantageous. A reduced or leuco dye is used to keep the substrate cytochrome *c* in the reduced state. The rate of oxidation is then measured by the rate of appearance of the oxidized dye color. The factors affecting this coupled reaction have been investigated and a standard assay technique has been proposed. Application of the method to rat tissues and pig heart muscle preparations has also revealed some new properties of the cytochrome oxidase system.

EXPERIMENTAL

Standard Method

Reagents and Apparatus—Cytochrome *c* was prepared from pig heart muscle according to Keilin and Hartree (3) but finally dialyzed against water. It was standardized spectrophotometrically, diluted to 2×10^{-4} M, and stored frozen. The dye, 2,6-dichlorobenzenoneindo-3′-chlorophenol (Eastman), was used without previous purification as in the previous oxidase methods (1, 2), but each lot of solid dye was standardized against ascorbic acid so that solutions of known molarity could be made by weight. Solutions of 35 mg. per cent (approximately 0.001 M) were prepared every 3 or 4 days. An amount of this stock dye solution for a day's use, usually 20 to 30 ml., is reduced as follows: (*a*) suspend in the dye solution by vigorous shaking about 0.5 mg. of 5 per cent palladized asbestos (Fisher) which had been previously thoroughly washed with water and oven-dried; (*b*) add 4 drops of 0.2 M McIlvaine buffer of pH 6.0 and bubble hydrogen through the solution until it is just decolorized; and (*c*) filter the mixture rapidly by suction through No. 5 Whatman paper and resume a slow stream of hydrogen to prevent autoxidation. As pointed out

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before (1), it is important that a minimal amount of catalyst be used and that the reduction not be carried beyond the point of decoloration.

The colorimetric measurements were made in a Lumetron colorimeter with a multiple reflection galvanometer, a 645 mμ filter, and an adapter for ½ × 4 inch test-tubes. This apparatus is convenient for transmission measurements at 5 second intervals and has adequate sensitivity for suspensions of high turbidity. The Beckman spectrophotometer was also used at the standard 3 ml. reaction volume as well as the 120 μl. level described later.

Procedure

The following technique was developed for tissue assay and was also used for the experimental work presented unless otherwise indicated. Further discussion of individual factors in the method will be found in subsequent sections.

Samples of 0.5 to 1.0 gm. of freshly excised tissue are blotted dry, weighed, cut into small pieces, and homogenized in 5 to 10 ml. of water in a Potter-Elvehjem homogenizer (4) in an ice bath. Homogenates are kept in an ice bath and assayed as soon as possible, suitable dilutions with water being made only immediately before assay and kept in the 30° bath only long enough for temperature equilibration, usually 3 minutes.

All reagents are kept in a 30° bath and the assay itself is completed before any significant change in temperature occurs in the reaction mixture. Reagents are added as follows: 1.0 ml. of 0.2 M phosphate buffer, pH 7.0, 0.5 ml. of 2×10^{-4} M cytochrome *c*, 1.0 ml. of 0.001 M leuco dye, and finally 0.5 ml. of enzyme suspension. The tube is quickly swirled for mixing and inserted in the colorimeter, the lamp rheostat is adjusted to a convenient galvanometer reading of 80 to 100, and transmission readings are taken at 5 second intervals usually in the period from 15 to 45 seconds after adding the enzyme. In this short time no evidence of change in transmission due to settling of particles was observed. The autoxidation rate of the leuco dye under these conditions is large enough to require correction and is measured by substituting water for the enzyme solution in the procedure. This rate is subtracted from those obtained in the presence of enzyme.

The per cent transmission (I) plotted against time on semilog paper gives linear curves for at least 30 to 60 seconds and the reaction rates are measured from the slopes of these curves. If curves are known to be linear, two readings are sufficient and plotting is unnecessary. Since the blank transmission (I_0) does not change significantly during the reaction, the rate of increase in optical density (D) and, by Beer's law, the rate of increase in oxidized dye concentration (C) are measured from the slope of the curve, as follows:

$$\frac{\Delta \log I}{\Delta T} = \frac{\Delta D}{\Delta T} = \frac{(\epsilon)(L)\Delta C}{\Delta T}$$

where T is in minutes. Reaction rates in this paper are expressed as $\Delta \log I$ per minute unless otherwise stated.

Enzyme activity in the conventional Q_{O_2} units, µl. of O_2 per hour per mg. dry weight, is calculated as follows:

$$Q_{O_2} = \frac{\Delta D \text{ per min. } (60)(3)(11.2)}{(29)(\text{mg. dry weight of preparation})} = \frac{\Delta D \text{ per min. } (70)}{\text{mg. dry weight of preparation}}$$

where 29 is the value of $(\epsilon)(L)$ for the dye and tubes used, and (60)(3)(11.2) converts µM per ml. per minute into µl. of gas per 3 ml. per hour.

Testing and Application of Method

Substrate System—The rate of oxidation of reduced cytochrome *c* itself can be used to measure oxidase activity (5, 6), but the coupled dye system appears to have several advantages. Preparation of the reduced dye is more convenient and the absorption peak of the oxidized dye is much broader than that of cytochrome *c* and more suitable for a filter instrument. The absorption measurement is also made at a much longer wave-length which avoids possible interference by other hemin compounds. In the Beckman spectrophotometer, where differences in the width of the absorption bands have a minimal effect on the absorption coefficients, cytochrome *c* has a somewhat higher change in coefficient during oxidation on an equivalent basis (20 sq. cm. per micromole) than the dye (15 sq. cm. per micromole). However, in rate measurements in the Beckman spectrophotometer, the dye-coupled substrate system always showed rates ($\Delta \log I$ per minute) equal to or greater than those of the cytochrome *c* system. The reason for this is not clear, although it may be mentioned that when cytochrome *c* is used hydrogen reduction was never complete, and with dithionite reduction there was some question of enzyme inhibition by dithionite oxidation products.

The same dye, 2,6-dichlorobenzenoneindo-3′-chlorophenol, used in the phenol oxidase (1) and peroxidase (2) methods, was chosen as the most suitable. The leuco dye concentration necessary to produce a maximum rate varied somewhat among homogenates of different tissues as indicated in Table I, but with all the preparations investigated 1 µM of leuco dye in the 3 ml. of reaction mixture was sufficient for "saturation." The rate of autoxidation was roughly proportional to the leuco dye concentration and this was not appreciably different in the presence of heat-inactivated enzyme.

As recorded in Table I, the tissue homogenates showed considerable variation with respect to the levels of cytochrome *c* required to produce a maximum rate. A partially purified heart muscle preparation gave a Michaelis constant of 6×10^{-6} M cytochrome in this system, similar to that found by Stotz, Altschul, and Hogness (7) for the hydroquinone system, but homogenates varied considerably. Kidney homogenates were the only ones encountered which were not saturated by the 0.1 μM of cytochrome *c* employed in the standard technique.

Effect of pH and Salt Concentration—Cytochrome oxidase activity was found to increase to a small extent from pH 6.5 to 7.5, but at pH values above 7.0 autoxidation of the leuco dye became too rapid for practical use. A pH of 7.0 gives the full blue color of the dye and was adopted for the standard method.

TABLE I

Effect of Leuco Dye and Cytochrome c Concentrations on Cytochrome Oxidase Activities of Rat Organ Homogenates

Rates expressed as Δ log *I* per minute.

Homogenates	Leuco dye, 35 mg. per cent			Cytochrome *c*, 2×10^{-4} M		
	0.5 ml.	1.0 ml.	1.5 ml.	0.25 ml.	0.50 ml.	0.75 ml.
Heart	0.28	0.29	0.29	0.31	0.38	0.37
Liver	0.48	0.51	0.52	0.72	0.97	1.01
Kidney	0.28	0.38	0.41	0.40	0.47	0.54
Intestine	0.52	0.67	0.65	0.41	0.47	0.47

Oxidase preparations showed a more pronounced and characteristic response to salt concentration. Fig. 1 illustrates the effect of phosphate buffer and NaCl concentration on the activity of various preparations. All showed a distinct maximum at 0.06 to 0.07 M phosphate and at about 0.11 M NaCl. Salt effects are not restricted to the system used in this paper. As early as 1912 Battelli and Stern (8) reported that the *p*-phenylenediamine-oxidizing system in brain mince increased about 35 per cent in activity with the addition of 0.1 to 0.17 M NaCl and that liver mince showed an optimal activity between 0.05 and 0.1 M. Elliott and coworkers (9, 10) have found that the respiration of liver and brain homogenates also was optimal when hypotonic preparations were made up to 0.08 to 0.1 M NaCl. They attributed part of the effect to osmotic action on cell integrity and part to action on the enzymes themselves. Recent evidence (11), however, has indicated that homogenates of the type used are essentially cell-free; hence presumably the oxidase effect observed and illustrated in Fig. 1 is mainly on the enzyme system. It seems clear that optimal activity of the cytochrome oxidase system depends upon an osmotic concentration in the range of 0.08 to 0.12 M, and M/15 phosphate buffer was adopted for routine use.

Low concentrations of aluminum ions have been used in cytochrome oxidase assays (12, 13), but in the present system with either fresh or aged homogenates no stimulating effect of aluminum was found.

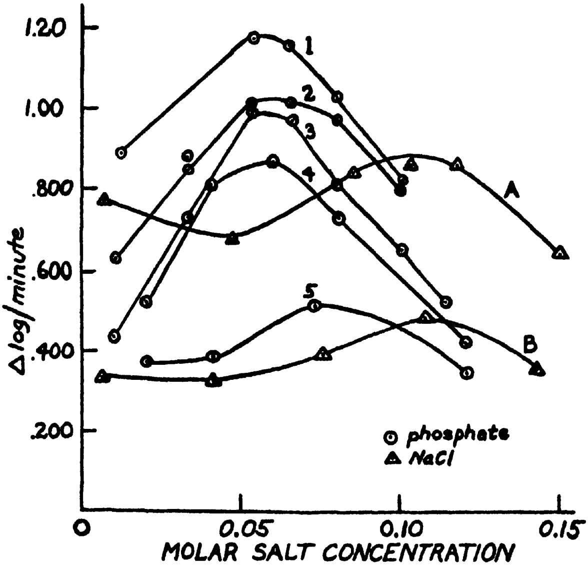

Fig. 1. The effect of salt and phosphate buffer on the activity of various cytochrome oxidase preparations. O, phosphate buffer curves. Curve 1, kidney homogenate; Curve 2, Keilin type of heart extract; Curve 3, ammonium sulfate-precipitated fraction of cholate clarified, Keilin type of heart extract; Curve 4, heart homogenate; Curve 5, liver homogenate. Δ, NaCl curves. Curve A, Keilin type preparation; Curve B, heart homogenate.

Relation of Enzyme Concentration and Rate of Reaction—Representative data in Table II show that under the standard assay conditions adopted reaction rates were directly proportional to enzyme concentration up to Δ log *I* per minute values of at least 1.50. This was true of both whole homogenates of several tissues and of partially purified heart muscle preparations. Under the conditions of the test described, a Δ log *I* per minute of 1.50 appears to be the safe upper limit for rate measurements, since in several instances rates of 2.0 or higher fell below the linear curve relating enzyme concentration and rate.

Enzyme Preparation—The preparation and preservation of the homogenate is critical in the assay of cytochrome oxidase. In our experience optimal activities were reached after 2 to 3 minutes of homogenizing with moderately tight fitting apparatus and with homogenate concentrations of 3 to 15 per cent. Such homogenates of rat organs rarely showed a change in activity during the first 2 hours of standing at 0°. However, after as little as 3 to 4 hours in some cases and consistently after standing overnight, the activities increased or decreased depending on the tissue. Six trials showed the following changes after 20 to 24 hours (average and range): heart 60 (35 to 120) per cent increase; liver, 34 (20 to 39) per cent decrease; large intestine, 27 (10 to 54) per cent decrease; lung, 30 (8 to 69) per cent decrease; and kidney, 45 (34 to 57) per cent decrease. Brain homogenates were inconsistent in stability, but usually increased in activity somewhat. These changes are avoided in practice by assaying homogenates within the 1st hour.

TABLE II

Effect of Enzyme Concentration on Rate of Leuco Dye Oxidation Measured under Standard Conditions

R, actual velocity measured as change in optical density per minute (ΔD per minute); R_1, calculated as the ratio of net velocity at the given enzyme concentration to that found at the relative enzyme concentration 5, the latter being arbitrarily assigned the R_1 value 5.00 (in bold-faced type).

Relative enzyme concentration	Rat heart homogenate		Heart oxidase*		Rat kidney homogenate		Rat liver homogenate	
	R	R_1	R	R_1	R	R_1	R	R_1
0	0.090†		0.048†		0.040†		0.070†	
1	0.182	0.94	0.216	1.04	0.280	1.10	0.340	1.06
2	0.278	1.92	0.352	1.90	0.464	1.94	0.630	2.11
5	0.580	**5.00**	0.852	**5.00**	1.14	**5.00**	1.35	**5.00**
10	1.06	9.90	1.64	9.85				

* Keilin type of phosphate extract from pig heart.

† This autoxidation blank subtracted from subsequent values in column to give "net velocity."

The stability of the diluted homogenates used in the colorimetric reaction is a more serious practical problem. Rat heart or liver homogenates, for example, when diluted from a 5 per cent suspension to 0.2 per cent, may lose 5 to 20 per cent of their oxidase activity in the first 5 minutes at 30°. The rate of deterioration varies considerably with the preparation and is much slower at 0°. The tendency with these two tissues is for the activity to become constant after 15 to 25 minutes at values of 50 to 75 per cent of the original. Attempts to stabilize these dilute homogenates at

30° with buffers, 0.5 per cent gelatin, or gum arabic were not effective. It is important therefore to carry out the assay as quickly as possible after diluting the stock homogenate at 30°. Further discussion of the problem of stability will appear later.

Reducing Material—Any substances in the enzyme preparation capable of reducing the oxidized dye could result in apparently lower oxidation rates in the oxidase assay. The presence of reducing material in the homogenate can be shown by measuring dye reduction in a system consisting of buffer, oxidized dye, and enzyme.

The rather difficult problem of measuring the rate of dye reduction, and therefore the extent of interference, which might occur during the oxidase assay was approached as follows. The usual test system was employed except that oxidized dye was present in a concentration at least equal to that which might possibly be present at the time of the first measurement in an oxidase assay, and the rate of reduction was measured. This concentration of oxidized dye was 0.25×10^{-4} M or some 10 per cent of the total dye employed in the oxidase assay. Under these conditions rat tissue homogenates gave reducing rates, expressed as per cent oxidizing rates, as follows: heart, 0 to 2; liver, 5 to 10; kidney, 2 to 10; brain, 5 to 15; intestine, 5 to 10; and lung, 10 to 15. There is reason to believe, however, that these estimates are too high. Thus it was found that the rate of reduction of a given amount of oxidized dye was less when a large proportion of reduced dye was also present, which is the situation in the oxidase assay. This was demonstrated with heat-inactivated oxidase preparations and with blood which contains similar reducing material but no oxidase. Furthermore, a significant interference by reducing material should manifest itself by a non-linear rate of dye oxidation in the oxidase test, since the rate of dye reduction increases with the concentration of oxidized dye, and the latter does increase progressively during the oxidase measurement. Yet the same linear rates of dye oxidation were found with preparations high in reducing materials as with those low in reducing power. A small error in the oxidase assay with preparations containing large amounts of reducing materials was nevertheless indicated by experiments in which the tissue preparation was first incubated for 30 to 60 seconds with a small amount of oxidized dye; cytochrome *c* and leuco dye were then added, and the rate of oxidation measured. With fresh homogenates of rat tissues the error due to reducing substances was considered to be too small to require correction, but the last method described for checking the extent of interference with other types of preparations is recommended.

Comparison of Colorimetric Method with Manometric Method—In addition to the obvious advantages of speed and convenience of the colorimetric assay it appears that the ability to perform the assay immediately after homogenization of the tissue leads to higher oxidase values than can be attained by the slower conventional manometric methods. Some differences in oxidase activity as expressed by Q_{O_2} values may be expected due to the different cytochrome reductants used in the tests, but the principal differences between manometric and the colorimetric method seem to be due to the time element in measuring the oxidase activity.

A variety of tissue preparations were studied both by the colorimetric method and by a manometric procedure similar to that described by Stotz (14), which employs hydroquinone as the reductant. The same pH, temperature, and concentrations of cytochrome *c* and phosphate buffer were employed in the two types of assay procedures. The results of both assays were expressed as Q_{O_2} values, and the ratio of the colorimetric Q_{O_2} to manometric Q_{O_2} calculated. Ratio values less than unity must represent the difference in the reductants employed in the two methods, while values greater than unity most obviously represent the deterioration of the oxidase preparations during the time necessary to set up the manometric experiment. Actually two series of comparisons were used, one (represented by (*b*) in Table III) in which the colorimetric assay was run during the first 5 minutes of the manometric measurement, and the second (represented by (*a*) in Table III) in which the colorimetric assay was performed within a few minutes after homogenization and dilution of the tissue.

TABLE III

Comparison of Q_{O_2} Values Determined Colorimetrically and Manometrically and Oxidase Activity of Rat Organs

Tissue homogenate	$\frac{Q_{O_2}\text{ colorimetrically}}{Q_{O_2}\text{ manometrically}}$					Q_{O_2} colorimetrically		
	No. of determinations	(*a*)*		(*b*)*		No. of determinations	Mean	S.E.†
		Range	Mean	Range	Mean			
Heart	6	0.97–2.02	1.29	0.56–0.85	0.72	11	244	15
Kidney	3	1.70–2.10	1.86	1.60–2.07	1.80	8	249	17
Brain	3	1.46–1.75	1.58	0.67–0.83	0.75	8	162	13
Liver	5	1.21–1.36	1.27	0.84–1.15	0.93	9	151	8
Large intestine	5	1.58–2.75	2.30	0.98–1.84	1.46	10	98	10
Lung	4	1.05–1.47	1.33	0.62–1.17	0.90	10	38.8	2.2
Heart oxidase‡	4	0.65–0.93	0.82	0.51–0.86	0.71			

* The ratios recorded under (*a*) are from colorimetric assays made immediately after homogenizing the tissue; those recorded under (*b*) from colorimetric assays made during the first 5 minutes of the manometric assay (see the text).

† Standard error $= \sqrt{\Sigma(x - \bar{x})/n(n - 1)}$.

‡ Keilin type of phosphate extract from pig heart.

The former is therefore a comparison in which the efficiency of the reductants should be most evident; the latter includes an expression of the stability of the oxidase in the preparations and is more strictly a comparison of the two types of assay in practice. Table III illustrates the results of typical experiments. In the (*b*) comparisons it will be noted that the manometric assays show slightly higher Q_{O_2} values with most of the tissue homogenates, although kidney and intestine homogenates show greater activity in the colorimetric assay. In the (*a*) comparisons all the tissue homogenates, except the relatively stable heart oxidase preparation, show a considerably greater oxidase activity in the colorimetric assay, apparently due to the instability of the oxidase in diluted tissue homogenates.

Table III also summarizes determinations by the colorimetric procedure on rat organs from unselected stock Wistar strain rats. The most striking difference in the relative oxidase activities of the tissues as determined by our method and by older methods (*cf.* Schneider and Potter (13)) is in the very high value found for kidney, although values for brain and liver are also somewhat higher.

Micro Modification—The colorimetric method is easily reduced in scale to small volumes, thus the following technique for oxidase analysis of histological sections has been devised for rat heart. Concentrations of the reagents were kept the same as in the macromethod described, but the cytochrome *c* and buffer were combined to give fewer volume measurements. The reaction mixture consisted of 40 μl. of leuco dye, 40 μl. of 1×10^{-4} M cytochrome *c* in 0.2 M phosphate buffer (pH 7.0), and 40 μl. of enzyme preparation, and the rate measurements were carried out in a constant temperature room with micro cuvettes and a Beckman spectophotometer adapted according to Lowry and Bessey (15). Rate curves were found to be as satisfactory as in the macrotechnique.

The tissue was prepared for testing by grinding frozen microtome sections, 2 mm. square and 10 μ thick, in 50 μl. of water in a small glass homogenizer. The chief difficulty was in preventing deterioration of the very dilute homogenate during grinding and subsequent work, which required that the grinder be kept ice-cold and that the assay be run as soon as possible after grinding. With this technique it was possible to measure the oxidase activity of slices weighing about 40 γ satisfactorily; hence the technique might be useful for localization of cytochrome oxidase in particular cell structures as has been done by Linderstrøm-Lang with arginase and peptidase.

DISCUSSION

The proposed colorimetric method for cytochrome oxidase has several advantages. It requires much less time than manometric methods, and is not only economical but also minimizes enzyme deterioration. It is considerably more sensitive on the same volume scale, and at the same time is more easily reduced in scale; thus at rates measured with comparable precision the colorimetric method requires one-third to one-fourth as much enzyme as the manometric with a reaction time of ½ minute instead of at least 15 minutes. For the same reaction time, the sensitivity of the colorimetric method is about 100 times as great. The colorimetric measurements can be made in any photoelectric instrument of sufficient sensitivity at a substantially lower cost in apparatus.

The relative oxidase activities of rat tissues as determined by the leuco dye method were somewhat different from those reported previously in which hydroquinone, ascorbic acid, or *p*-phenylenediamine were used as cytochrome reductants. Some of the differences are undoubtedly due to a greater instability of the oxidase in some tissue homogenates than others, but there were also variations among the other methods and no single reductant has an obvious theoretical advantage over the others, and each has its own practical advantages. It is not clear, in fact, why the added reductant should affect the rate, when it is present in excess, if its reaction rate with oxidized cytochrome *c* is not limiting, and if it or its oxidation product does not inhibit the enzyme. The latter is known to be a serious problem with hydroquinone, since the quinone is a powerful oxidase inhibitor. It is very unlikely that this is true of the leuco dye system, since large increases in the per cent of oxidized dye did not affect the rate as long as the system was kept saturated with leuco dye. In the colorimetric method only about one-sixtieth the concentration of the oxidized substrate is formed during the reaction as in the manometric method.

Experience with the new method applied to rat tissue homogenates and various pig heart muscle preparations has pointed strongly to the dependence of oxidase activity on the physical state of the particles with which it is associated. It has been shown at least with liver cells that the bulk of the oxidase is on the large granules (16, 17) and that the latter are very sensitive to changes in salt concentration as judged by microscopic appearance (18). Earlier reports and our own studies might be interpreted that the salt effect was on the oxidase itself or on the physical state of the particles which may alter the efficiency of the whole complex of enzymes associated with these structures. Another line of corroborative evidence comes from the effect of bile salts on oxidase preparations. Unpublished work in this laboratory has shown that low concentrations of cholate and desoxycholate cause up to 100 per cent increase in oxidase activity as determined either colorimetrically or manometrically. This effect seems to be correlated with the dispersing and clarifying action up to certain cholate levels, but above these levels inhibition occurs. The characteristic changes in the activities of homogenates on aging may result from physical changes in the particles. In several cases with liver preparations, in fact, these changes have been shown to be reversible. Stock homogenates which had dropped 25 to 30 per cent in activity overnight were found to rise again on dilution to about the original level after a few minutes at 30°.

The variation in relative oxidase activites among tissues with different substrates may also be associated with the physical state of the particles. As a result of the marked salt effect on the oxidase, its instability, and the dependence of activity on the physical state of the enzyme, cytochrome oxidase determinations must be rigidly controlled and are as yet highly empirical.

SUMMARY

A colorimetric method for the rapid determination of cytochrome oxidase is described in which the substrate, cytochrome *c*, is kept reduced by leuco-2,6-dichlorobenzenoneindo-3′-chlorophenol, and the rate of oxidation is measured photometrically by the appearance of oxidized dye color. Under the conditions described the rate is linear for 30 to 60 seconds and is directly proportional to enzyme concentration.

Optimal levels of leuco dye and cytochrome *c* were determined and it was found that oxidase preparations showed a distinct salt effect with optimal activity at osmotic concentrations of 0.08 to 0.12 M. Investigation of the preparation and preservation of homogenates emphasized the necessity for speed for which the present technique is well adapted. Comparison of manometric and the proposed colorimetric methods showed that considerable loss in oxidase activity may occur with the former. The new method, inherently more sensitive, has been further reduced in scale so that oxidase determinations may be made on small microtome sections of tissues.

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PROTECTION OF GLYCOLYSIS IN MOUSE BRAIN HOMOGENATES BY AMIDES AND ESTERS OF AMINO ACIDS*

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It has been shown previously that glycolysis of mouse brain homogenates is impaired in a characteristic manner by a variety of apparently unrelated substances. Certain neurotropic viruses, proteolytic enzymes, and ferrous or zinc salts markedly inhibit the utilization of glucose under specific conditions (1, 2). Because of the similarity between the effect of ferrous salts and that of proteolytic enzymes, the possibility was considered that a cathepsin-like enzyme may be present in mouse brain which is activated by iron salts and which in turn inactivates a glycolytic enzyme. Such an "inactivating factor" requiring iron salts and a reducing agent such as ascorbic acid was found in repeatedly washed and dialyzed particles of mouse brain homogenates. The glycolytic enzyme most rapidly inactivated by this factor was identified as glyceraldehyde phosphate dehydrogenase. Addition of crystalline preparations of this enzyme was found to restore glycolytic activity to mouse brain homogenates inactivated by either metal salts, neurotropic viruses, or proteolytic enzymes.

It was thought desirable to find a small molecular substance, either a peptide or an amino acid derivative, which would compete with glyceraldehyde phosphate dehydrogenase for the proteolytic enzyme (inactivating factor) and thus protect the glycolytic enzyme from inactivation. Since the inactivating factor of mouse brain showed similarities to cathepsin III with regard to its activation by reducing agents, L-leucinamide, the known synthetic substrate for this enzyme, was tested and found effective in protecting glycolysis in the presence of iron salts.

It is the purpose of this paper to report on the protective action of L-leucinamide and other amino acid derivatives on the glycolytic system of mouse brain and also to present further data on the iron-activated factor of brain and its relation to cathepsin III purified from beef spleen.

Methods

The preparation of actively glycolyzing mouse brain homogenates and of the necessary coenzymes was carried out as described previously (1, 2).

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Amino Acid Derivatives—Glycine ethyl ester and *N*-phenylglycine ethyl ester were commercially obtained preparations. The other esters of amino acids used in this study were prepared by treating the amino acids in absolute ethyl alcohol with dry HCl and evaporation of the esters to dryness *in vacuo*. The amide hydrochloride of L-leucine was prepared from its ester (3) and recrystallized from alcohol-benzene.

Commercially obtained amino acids as well as the esters and amides prepared from them contained iron salts as contaminants which were readily detectable by the α,α'-dipyridyl color reaction. To remove the iron salts, the amides were dissolved in water and treated with an excess of 8-hydroxyquinoline which was then removed by repeated extraction with chloroform. The solvent was removed by evaporating the amide solution to dryness *in vacuo*. The iron salts were removed from the esters by the addition of equivalent amounts of calcium chloride and phosphate buffer at pH 7.8. The mixture was warmed to 80° and filtered (4). Occasionally a short treatment of the mixture with hydrogen sulfide was necessary to facilitate removal of the iron.

Cathepsin III—The preparation of cathepsin III from beef spleen was carried out according to the method described by Fruton and Bergmann (5). The preparation was further purified by the following procedure: The ammonium sulfate paste obtained by the method of Fruton and Bergmann (5) was dissolved and dialyzed against 1 per cent potassium chloride overnight. The precipitate which formed was removed by centrifugation and discarded. The supernatant was adjusted to pH 4.5, heated rapidly to 60° by placing it in a water bath at 80° with vigorous continuous stirring, and then maintained in a 60° water bath for 5 minutes. After rapid cooling the mixture was filtered and the precipitate discarded.

No loss of activity occurred during this procedure. On the contrary, quite consistently an increase in total activity was obtained, probably due to the removal of an inhibitor. The supernatant was then dialyzed against 1 per cent KCl at pH 4.5 for 3 hours with mechanical stirring and was again centrifuged. The clear supernatant was fractionated with alcohol at −6°. The fraction precipitated by alcohol concentration of 30 per cent was collected. Occasionally it was observed that the fraction obtained between 30 and 45 per cent alcohol concentration showed a higher specific activity than that of the 30 per cent alcohol precipitate. For most of the experiments described in this paper, the fraction precipitated at 30 per cent alcohol was used after dialysis for 1 hour with stirring against 1 per cent KCl at 2°. The preparations lose activity on storage even at 0°; however, they retain undiminished activity for several months when stored in a dry-ice box at −70°.

Inactivation Factor (*IF*)—In most experiments the preparations of the inactivating factor used were obtained by homogenizing mouse brains in 10 volumes of 0.01 M potassium phosphate buffer of pH 7.4 at 0°, centrifuging, and washing the particles twice with the same buffer in the cold. The washed particles were suspended in one-fourth the original volume of the homogenate and 0.2 to 0.4 ml. was used to inhibit glycolysis. To remove iron salts the preparation was treated as described below.

Determination of Enzymatic Activity—The enzymatic hydrolysis of amides of amino acids was measured by two methods. In earlier experiments the formol titration method (6) with phenolphthalein as indicator or potentiometric measurements were used. Later a quantitative ninhydrin reaction (7) was employed to determine the hydrolysis of L-leucinamide. Since with this method both products of the hydrolytic reaction, L-leucine and ammonia, give the color reaction, the sensitivity of the reaction is nearly doubled. Corrections for residual L-leucinamide after hydrolysis were made. Since the amide gives only a small fraction of the color intensity obtained with either L-leucine or ammonia, the correction factor is small.

The hydrolysis of the amino acid esters was followed potentiometrically (8), as will be described later in this paper.

Results

Properties of Inactivating Factor (IF) Present in Mouse Brain—In fresh mouse brain homogenates the factor which is responsible for the inhibition of glycolysis requires the addition of iron salts for its activation. All attempts to purify IF resulted at first in spontaneous activation, so that addition of iron salts was no longer necessary. Spontaneous activation of IF is probably due to the release of iron from tissue components. The spontaneous activation is reversible, since addition of iron salts is again required following dialysis against hydrocyanic acid at pH 6.5. To remove iron completely, dialysis for 4 to 5 days with frequent changing of the hydrocyanic acid is required. The cyanide is then removed by further dialysis against 0.01 M potassium phosphate at pH 6.5. To reactivate preparations rendered iron-free by dialysis against cyanide requires addition not only of iron but also of cysteine or ascorbic acid (see Table I). Dialysis at neutral or slightly alkaline pH resulted in inactivation which was no longer reversed by the addition of iron salts and reducing agents.

It has been shown previously (2) that, in order to obtain reproducible inhibition of glycolysis by iron salts, a preliminary period of incubation of the brain homogenate in the presence of iron salts is required (20 minutes at 38°). After this period, the coenzymes are added to start glycolytic activity. It can be seen from the data shown in Table I that IF prepared as described above, activated by iron salts and ascorbic acid, exerts a direct inhibitory effect on glycolysis without preliminary incubation of the brain homogenate. Full activation of this cathepsin-like enzyme is achieved only by addition of both ferrous sulfate and ascorbic acid. Ferrous sulfate or ascorbic acid alone does not fully activate the inactivating factor. Ferrous sulfate and ascorbic acid added without IF have little or no effect on glycolysis of brain homogenates.

Attempts at purification of IF did not yield consistent results. Sonic vibration, salt extraction, use of protamine, and lyophilization failed to yield reproducible preparations of soluble inactivating factor.

TABLE I

Effect of Ferrous Sulfate and Ascorbic Acid on Inactivating Factor

0.3 ml. of brain homogenate in 0.01 M potassium phosphate of pH 7.4; 0.1 ml. of 0.24 M glucose; 0.1 ml. of 0.16 M $KHCO_3$; 0.1 ml. of 0.2 M ammonium phosphate of pH 7.6; 0.1 ml. of 0.07 M $MgCl_2$; 0.1 ml. of 0.01 M adenosine triphosphate; 0.1 ml. of 4 per cent nicotinic acid amide; 0.1 ml. of 0.8 per cent diphosphopyridine nucleotide (60 per cent purity). Incubated for 60 minutes at 38°. 0.1 ml. of 0.005 per cent of ferrous sulfate·$7H_2O$ and 0.1 ml. of 0.15 per cent ascorbic acid neutralized before use were added to 0.2 ml. of the inactivating factor when indicated.

Tissue preparation	Addition	Lactic acid produced	Inhibition of glycolysis
		mg.	*per cent*
Brain homogenate		2.2	
" "	Ferrous sulfate	2.2	
" "	Ascorbic acid	2.0	9.0
" "	" " + ferrous sulfate	2.0	9.0
" " + inactivating factor	Ferrous sulfate	1.75	20.0
" " + " "	Ascorbic acid	1.25	43.0
" " + " "	" " + ferrous sulfate	0.45	80.0

Similarities of Inactivating Factor to Cathepsin III—The inactivating factor of mouse brain has been shown to affect the enzyme proteins glyceraldehyde phosphate dehydrogenase and phosphofructokinase (2). A proteolytic change was considered, therefore, as one of the likely possibilities. The hypothesis was further strengthened by the observation that certain proteolytic enzymes exert an effect similar to that of IF (2). Furthermore, preparations of the inactivating factor are stable when dialyzed at an acid pH but lose activity at an alkaline pH in a manner similar to the tissue cathepsins. In the studies of Fruton and Bergmann (5) the splitting of synthetic substrates by cathepsins and the activation of these enzymes by either ascorbic acid or cysteine was used as a basis for classification. Thus, cathepsin I required neither cysteine nor ascorbic acid, cathepsin II required cysteine and was not activated by ascorbic acid, and cathepsin III could be activated by either cysteine or ascorbic acid. The specific substrate used by Fruton and Bergmann for cathepsin III was L-leucinamide.

The inactivating factor of mouse brain is activated by either cysteine or ascorbic acid and in this respect it resembles cathepsin III. Furthermore, preparations of cathepsin III when added to normal mouse brain homogenate cause an inhibition of glycolysis similar to that observed with IF (2). For these reasons, a comparative study of cathepsin III and IF was carried out.

Cathepsin III was purified from beef spleen as described above. The preparation still contained considerable amounts of bound iron which could not be removed on dialysis. The addition of α,α'-dipyridyl to the cathepsin preparation resulted in pronounced inhibition of L-leucinamide hydrolysis. Inhibition by α,α'-dipyridyl has been used (9) to demonstrate activation of enzymes by metals. Thus, a participation of metals in the activity of spleen cathepsin III is suggested by these results.

Attempts were made, therefore, to remove the iron salts from the cathepsin preparations. Successful removal of the iron salts was accomplished by dialysis against α,α'-dipyridyl and a reducing agent. The treatment, however, resulted in irreversible inactivation of the cathepsin and all attempts to reactivate the enzyme by means of iron salts and reducing agents failed.

Effect of Amides and Esters of Amino Acids on Inhibition of Glycolysis by Ferrous Sulfate—Because of the similarities of cathepsin III and the inactivating factor of brain the effect of L-leucinamide on brain glycolysis was tested. It was hoped that a substance which is attacked as slowly as the synthetic amides might have an inhibitory effect on proteolysis by competition for the enzyme and thus protect the glycolytic enzymes. It was found that addition of L-leucinamide to brain homogenate protected the glycolytic activity from the inhibition by iron salts. On the other hand glycylleucinamide, leucine peptides, glutamine, and asparagine were found inactive. Benzoylargininamide, generally used as a substrate for trypsin, had only slight protective activity. Leucine ethyl ester and some other amino acid esters, on the other hand, were found to be fully as effective as L-leucinamide. A list of substances tested is shown in Table II. It may be noted that *N*-phenylglycine ethyl ester was the most active of the compounds tested.

The possibility was considered that L-leucinamide and the other active compounds might exert their protective effect by binding the iron and thus preventing its action. However, a 5-fold increase in ferrous sulfate concentration did not alter the protective effect of L-leucinamide when the latter was used in suboptimal concentration. This finding makes it unlikely that a direct interaction between iron salts and L-leucinamide is responsible for the protective action. Moreover, when cathepsin III was added directly to glycolyzing brain homogenates, a partial protection was obtained with L-leucine ethyl ester (Table III).

TABLE II

Protective Effect of Peptides, Amides, and Esters of Amino Acids on Glycolysis of Brain Homogenates

The experimental conditions were as in Table I except that the brain homogenates were incubated for 20 minutes at 38° in the presence of 0.1 ml. of 0.24 M glucose, 0.1 ml. of 0.005 per cent ferrous sulfate·$7H_2O$, and the peptides, amides, and esters of the amino acids tested. After this preliminary incubation, the buffers and coenzymes were added and the mixture reincubated for 60 minutes.

Compounds tested		Protective activity
Peptides	Glycylglycine, leucylglycine	Inactive
	Glycylleucine, glycylserine	"
	Glycylasparagine	"
	Leucylglycylglycylglycine	"
	D-Alanylglycine	"
	Glutathione	Very active
Amides	Leucinamide	Active
	Benzoylargininamide	Weakly active
	Glutamine, asparagine	Inactive
	Malonamide	Weakly active
	Urea	Inactive
	Glycylleucinamide	"
	Carbobenzoxyglycylleucinamide	"
Esters	Leucine ethyl ester	Active
	Valine ethyl ester	"
	Glycine ethyl ester	"
	Glycylglycine ethyl ester	"
	Cysteine ethyl ester	"
	N-Phenylglycine ethyl ester	Very active

Unless specified the L-amino acid derivative was tested. We wish to thank Dr. M. Levy for his generous gift of the peptides listed and Dr. E. Smith for the glycylleucinamide and the carbobenzoxyglycylleucinamide.

A similar though less pronounced protection of glycolysis by the same amino acid esters, among which *N*-phenylglycine ethyl ester was the most active, was obtained in brain homogenates of mice infected with Theiler FA mouse encephalomyelitis virus.

Esterase Activity of Cathepsin III Preparations—In using the esters of amino acids, it was hoped they would act as competitive inhibitors rather than as substrates for the proteolytic enzymes. At the time these experiments were in progress, Schwert, Neurath, Kaufman, and Snoke (8) reported that crystalline preparations of trypsin possessed very high activity in catalyzing the splitting of α-benzoyl-L-arginine methyl ester and provided convincing evidence for the amidase and esterase activity of trypsin.

It was of interest, therefore, to test the possibility that the esters of the amino acids used in the present study might be split by cathepsin preparations and thus act as substrates rather than as non-hydrolyzable competitive inhibitors.

It was found that the crude brain homogenates as well as the purified cathepsin preparation readily hydrolyze L-leucine ethyl ester. The esterase activity tests were carried out with a Beckman model G pH meter, essentially as described by Schwert *et al.* (8). Citrate buffer in 0.005 M final concentration was used at pH 5.3 as the null value. Ascorbic acid was always added to give a 0.04 M concentration. To test the effect of iron salts, 1.8×10^{-5} M ferrous sulfate was added. The substrate concentration was 0.05 M. With this excess amount of L-leucine ester the rate of hydrolysis followed a zero order reaction and was proportional to the amount of enzyme added (see Fig. 1). The enzyme preparation of cathepsin III used in this experiment was purified as described above.

TABLE III

Protective Effect of Leucine Ethyl Ester on Glycolysis by Brain Homogenates

The experimental conditions are as in Table II. Addition of about 3 to 5 mg. of the purified cathepsin preparations was required to give pronounced inhibition of brain glycolysis. 0.3 ml. of a 1 per cent solution of leucine ethyl ester was added when indicated.

Tissue preparation	Addition	Lactic acid produced	Inhibition of glycolysis
		mg.	*per cent*
Brain homogenate		2.0	
" "	Leucine ethyl ester	2.0	
" " + ferrous sulfate		0.8	60.0
" " + " "	Leucine ethyl ester	1.5	25.0
" " + cathepsin III		0.45	77.0
" " + " "	Leucine ethyl ester	1.25	37.0

This rapid and convenient test system was used to follow the esterase activity during purification. At several stages in the procedure comparison of the esterase activity with the activity in splitting L-leucinamide was made. The esterase and the amidase activity were not separable by fractionation and the specific activity against both substrates was increased to the same extent during the process of purification. The pH optimum of the esterase activity is approximately 5.4 (see Fig. 2). The same optimum is reported by Fruton and Bergmann for the amidase activity. The Michaelis constant for the esterase activity with L-leucine ester as substrate is 1.1×10^{-2}. Both the esterase and amidase activities are destroyed by temperatures above 60° but withstand this temperature for 5 minutes without apparent loss of activity. Both esterase and amidase are activated by ascorbic acid. From this evidence it appears likely that cathepsin, in analogy to trypsin (8), has esterase as well as proteolytic activity. However, in the case of cathepsin, definite proof for this is lacking, since the enzyme preparations are still impure.

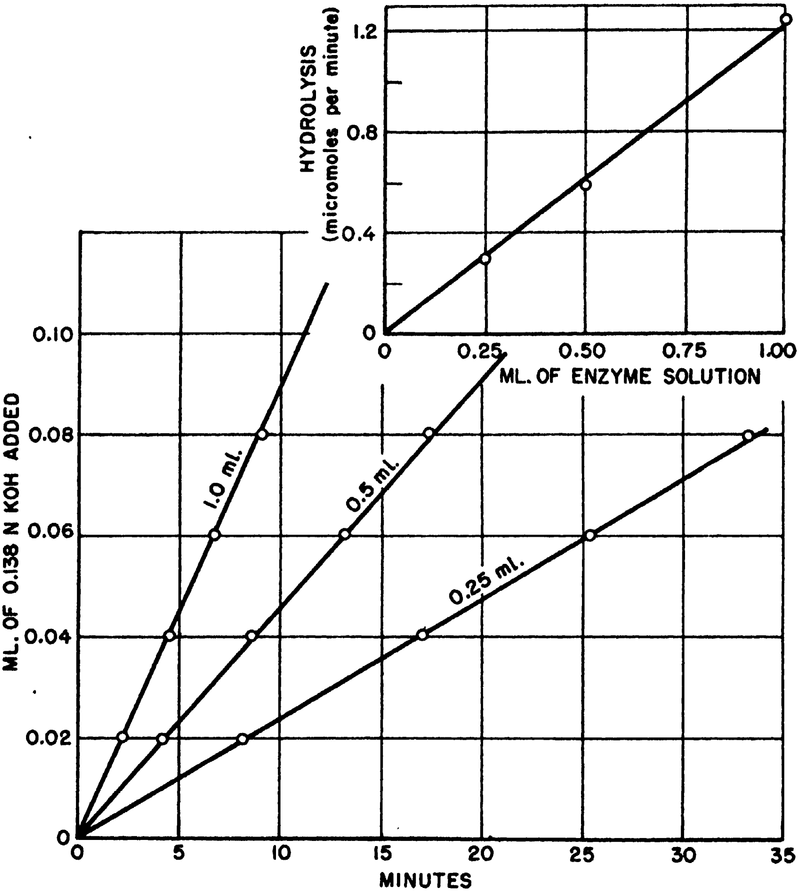

FIG. 1. Esterase activity of cathepsin III preparations from beef spleen. Measurements as described in the text at pH 5.4; hydrolysis of leucine ethyl ester by various concentrations of cathepsin III; temperature 25°.

Substrate Specificity of Esterase—In the search for competitive inhibitors of proteolysis in brain homogenate a number of esters were tested. These

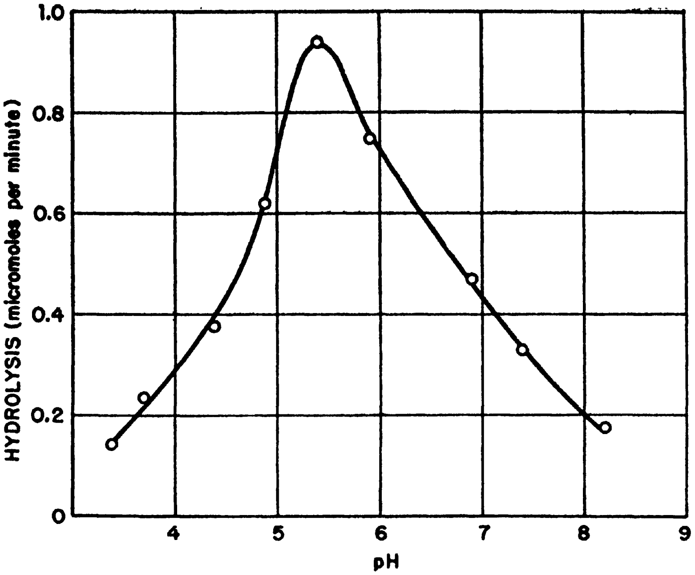

FIG. 2. Effect of pH on hydrolysis of cysteine ethyl ester (0.05 M) by cathepsin III preparations.

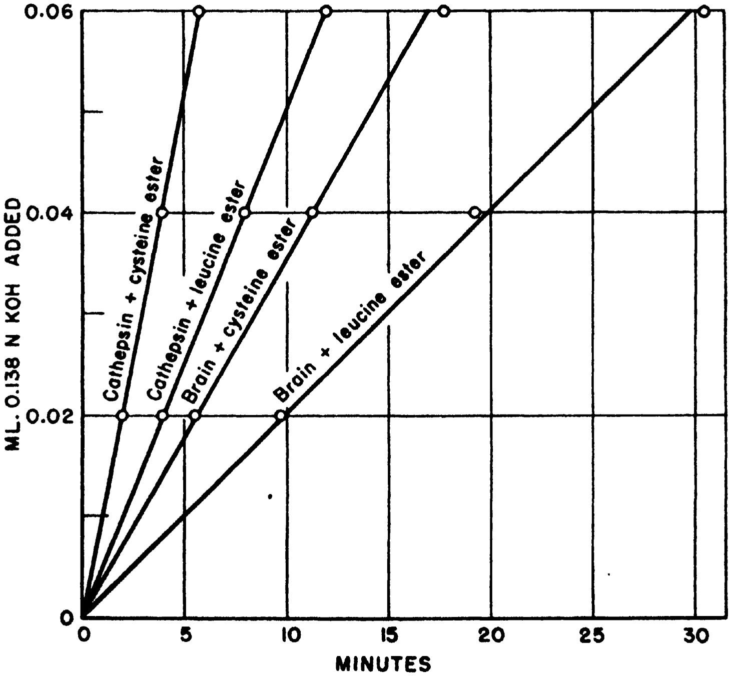

FIG. 3. Comparison of hydrolysis of cysteine ethyl ester and leucine ethyl ester by spleen cathepsin preparations (pH 5.4) and brain homogenates (pH 7.4).

esters were now tested as possible substrates for the cathepsin III preparation. Of these, cysteine ethyl ester was found to be more rapidly hydrolyzed than L-leucine ester. Glycine ethyl ester was also hydrolyzed by the cathepsin III preparation but at a slower rate. The other esters listed in Table II showed little or no hydrolysis by the enzyme preparation.

Esterase Activity of Brain Homogenates—Since the studies of glycolysis were carried out at pH 7.4, the effect of fresh mouse brain homogenates was tested at this pH on some of the amino acid esters. Cysteine ethyl ester was hydrolyzed most rapidly and L-leucine ethyl ester was split at about half the rate. The results are recorded in Fig. 3, and comparative data with spleen cathepsin are also shown.

DISCUSSION

It has been postulated (2) that the inhibitory effect of iron salts and certain neurotropic viruses on glycolysis is due to the activation of a proteolytic enzyme present in brain homogenates. This hypothesis was based on similarities between the inhibitory effect of iron salts on glycolysis and that exerted by certain proteolytic enzymes. In either case it was possible to restore the glycolytic activity by addition of crystalline glyceraldehyde phosphate dehydrogenase. The evidence presented in this paper seems to strengthen further the hypothesis that the inhibition of glycolysis is due to proteolysis. The iron-activated factor purified from mouse brain homogenates is in several characteristics similar to a tissue cathepsin. It is stable at acid and unstable at alkaline pH and is activated by reducing agents such as cysteine and ascorbic acid.

The latter observation led to a comparative study of the inactivating factor and cathepsin III, an enzyme purified from beef spleen by Fruton and Bergmann (5). Cathepsin III, like the inactivating factor of mouse brain, is activated by cysteine or ascorbic acid. The synthetic substrate used by Fruton and Bergmann for cathepsin III was L-leucinamide. It has been shown in the present study that L-leucinamide or L-leucine ethyl ester effectively protects glycolysis in the presence of ferrous sulfate or in the presence of cathepsin III, presumably by competing with the glyceraldehyde phosphate dehydrogenase for the active center on the cathepsin.

The sequence of events occurring in the inhibition of glycolysis in brain homogenates may be described as follows. Inhibition of glycolysis by ferrous sulfate, neurotropic viruses, or added cathepsin is due to the destruction of glyceraldehyde phosphate dehydrogenase which is the key enzyme of coupled phosphorylation (2). In the case of added cathepsin III preparations, it is believed that glyceraldehyde phosphate dehydrogenase is inactivated directly by the proteolytic action of cathepsin III. In the case of iron salts or the neurotropic viruses a factor present in homogenates of normal mouse brain is activated which then behaves like a cathepsin and destroys glyceraldehyde phosphate dehydrogenase. The action of some neurotropic viruses has been explained previously by the presence of considerable amounts of non-dialyzable iron in the purified virus preparations (1).

When tissues are homogenized for the purpose of metabolic studies the destruction of cells leads to disruption of the spatial relationship between enzymes and coenzymes and their substrates. Proteolytic enzymes are released as well as enzymes capable of destroying coenzymes. This has been well recognized in the past few years. The destruction of coenzymes such as diphosphopyridine nucleotide, triphosphopyridine nucleotide, adenosine triphosphate, and flavin-adenine dinucleotide is known to account for many failures to obtain enzymatic activity in homogenates. Nicotinic acid amide can be used to protect the pyridine nucleotides and fluoride to delay the destruction of adenosine triphosphate.

It has been shown in the present studies that in brain homogenates effective protection for the protein substrate, the enzyme glyceraldehyde phosphate dehydrogenase, can be obtained by the use of certain substrates or competitive inhibitors of low molecular weight. The protective effect of esters and amides of amino acids against the proteolytic destruction of a glycolytic enzyme is probably analogous to the inhibition of cozymase destruction by nicotinic acid amide. While some of the esters and amides seem to be destroyed during the process, others, such as phenylglycine ethyl ester, are not hydrolyzed. Perhaps this fact explains why the latter compound is effective in much lower concentration than L-leucine ester which is itself hydrolyzed.

The specificity of the protective effect of certain amino acid esters and amides is difficult to appraise at the present time. The structural resemblance between some of the active compounds is remote, while other more closely related compounds are inactive. Further study, it is hoped, will determine whether or not the protective effect of the various esters is due to protection of the same or of different structures of the protein molecule.

Some of the esters which protect the glycolytic process from inactivation by spleen cathepsin or from the iron salt inhibition are also effective in partially protecting against the inhibition observed in homogenates of the brains of mice infected with Theiler FA mouse encephalomyelitis virus. The investigations described in this and previous papers were carried out with the purpose of shedding light on the mechanism of neuron destruction during infection by certain neurotropic viruses. *In vitro* studies showed inhibition of glycolysis in the presence of virus which was traced to the destruction of the enzymatic activity of glyceraldehyde phosphate dehydrogenase. Circumstantial evidence points to activation of a proteolytic enzyme by the virus, although it should be emphasized that final proof for a proteolytic process is lacking. It is hoped that the protection by the amino acid esters, if a more effective competitive inhibitor can be found, may serve as a tool for appraising the rôle which the inhibition of glucose metabolism plays during the infectious process.

SUMMARY

1. The glycolytic activity of mouse brain homogenates is inhibited by an inactivating factor present in normal brain. This factor requires Fe^{++} and either cysteine or ascorbic acid as activators.

2. A similar inhibition of glycolysis is produced by cathepsin III purified from beef spleen.

3. The inhibition of glycolysis produced by either a cathepsin III preparation or the inactivating factor of brain is prevented by the addition of certain amides and esters of amino acids.

4. Cathepsin III preparations as well as homogenates of mouse brain hydrolyze the ethyl esters of L-leucine and L-cysteine.

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MICROESTIMATION OF α-AMINO ACIDS WITH *peri*-NAPHTHINDAN-2,3,4-TRIONE HYDRATE

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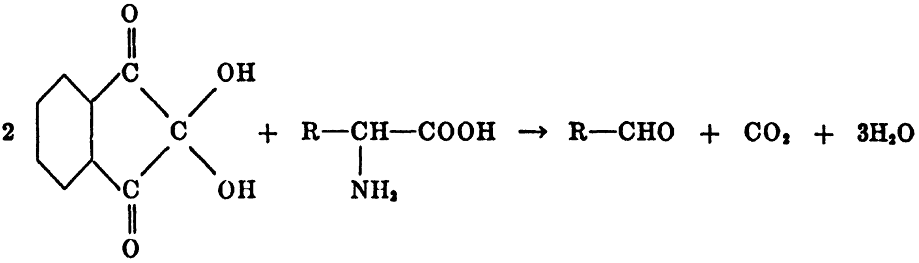
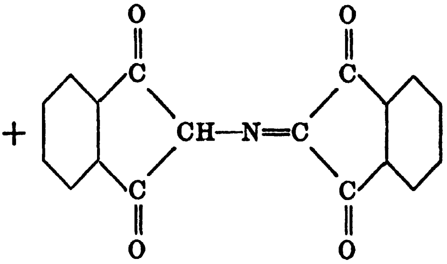
When free α-amino acids are heated with triketohydrindene hydrate (ninhydrin) (I) in neutral or slightly acidic medium, there are formed the corresponding aldehydes with 1 carbon atom less, ammonia, carbon dioxide, and diketohydrindylidenediketohydrindamine (II) (*cf.* Ruhemann (1)).



(I)

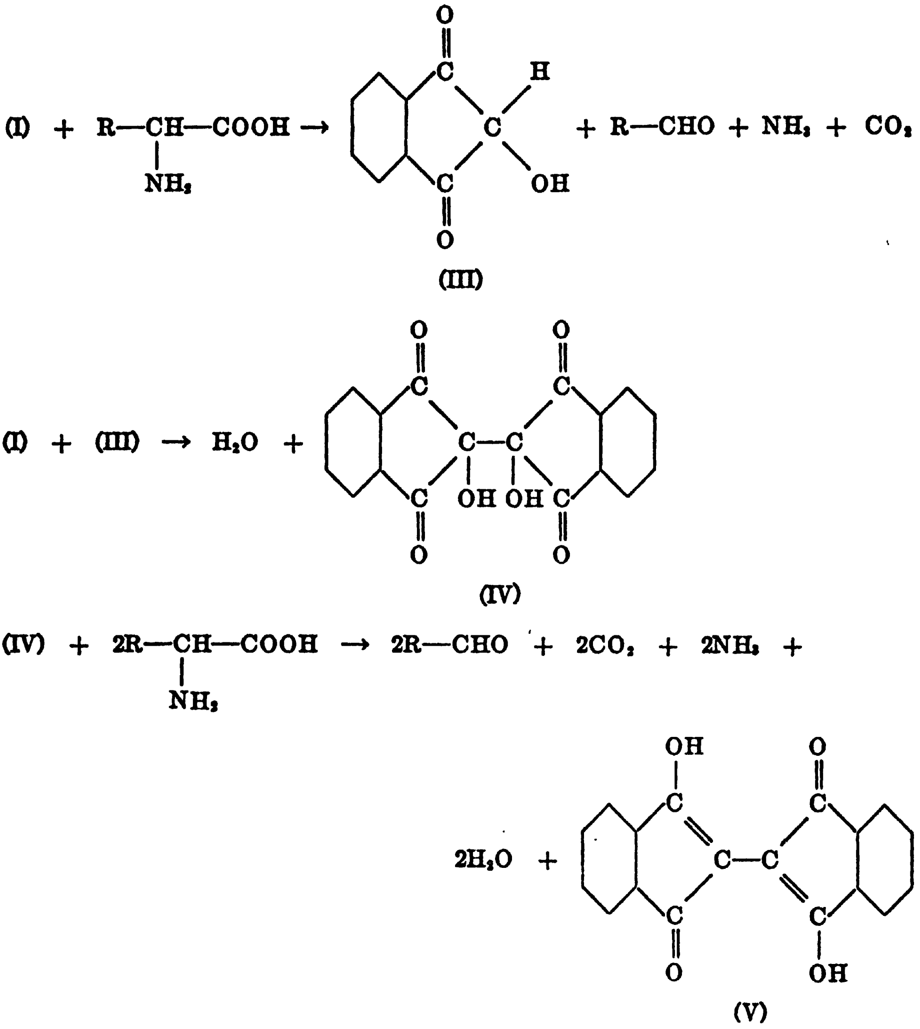


(II)

Recently, Moubasher and Mostafa (2) found that the reaction between ninhydrin and α-amino acids proceeds differently from that mentioned above, as two different compounds have been isolated, namely hydrindantin (IV) and bis-1,3-diketoindane (V), according to the accompanying scheme.

Virtanen, Laine, and Toivonen (3) have described a quantitative method for the estimation of α-amino acids in protein hydrolysates based on the quantity of aldehyde liberated by means of ninhydrin. A method for the determination of free α-amino acids by titration of the carbon dioxide formed in the reaction with ninhydrin has been described by Van Slyke, MacFadyen, and Hamilton (4). It was also found that isatin in glacial acetic acid and chloramine-T at pH 2.5 may be used in place of ninhydrin (*cf.* Van Slyke, Dillon, MacFadyen, and Hamilton (5)).

Moubasher (6) has used *peri*-naphthindan-2,3,4-trione hydrate (VI) as a new reagent for the quantitative determination of α-amino acids, as this reagent is able to decompose the α-amino acids quantitatively to the next lower aldehyde with 1 carbon atom less with the formation of ammonia, carbon dioxide, and dihydroxy-*peri*-naphthindenone (VII).



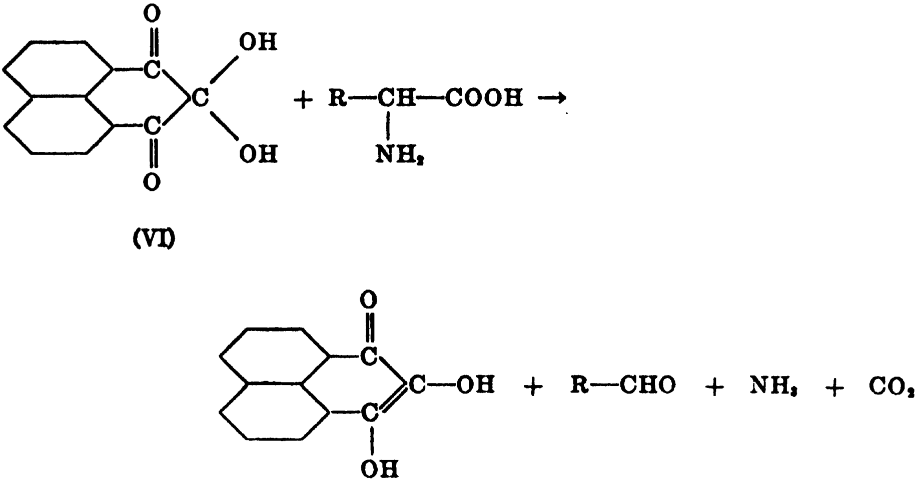
(III)

(IV)

(V)

Wertlind (7) has developed a method for the microestimation of valine by its degradation with ninhydrin to isobutyraldehyde. The aldehyde is steam-distilled and then determined colorimetrically by Fabinyi's reagent (8). It is not stated in Wertlind's paper whether the Fabinyi reaction is given by the volatile aldehydes arising from other amino acids. We have found that acetaldehyde, isovaleraldehyde, and benzaldehyde, which are formed through the degradation of alanine, aspartic acid, leucine, and phenylaminoacetic acid respectively, give Fabinyi's reaction.

In the present work it is shown that *peri*-naphthindan-2,3,4-trione hydrate may be used in place of ninhydrin for the degradation of alanine, valine, leucine, aspartic acid, and phenylaminoacetic acid, to their corresponding aldehydes with 1 carbon atom less according to the accompanying scheme (*cf.* Schönberg, Moubasher, and Mostafa (9), Moubasher (6), Moubasher and Awad (10)).



(VI)

(VII)

In order to avoid possible loss of the distilling aldehyde, this quantitative degradation of α-amino acids to the corresponding aldehydes and the steam distillation are carried out in one apparatus in place of the two used by Wertlind. The apparatus is similar to that described by Virtanen *et al.* (3). The procedure may be adapted to the microestimation of the aldehydes mentioned above.

EXPERIMENTAL

Apparatus—

1. Universal colorimeter (Ernst Leitz), illuminated by its ordinary electric lamp.

2. Micro pipette of 1 cc. capacity.

3. Reaction and distilling apparatus. A distillation flask (*A*) (10 cc. capacity) is attached to a separatory funnel (*B*) by a ground joint. The neck of the distilling flask is adapted with a capillary tube (*C*) by means of a

ground joint, through which a continuous current of carbon dioxide is passed during the reaction. *D* is a bulb condenser attached to the neck of the flask through a ground joint. *E* is a bulb fitted to the condenser by a ground joint, the end of which is dipped in a test-tube (*F*), strongly cooled from the outside by an ice-salt mixture.

Reagents—

1. Citrate buffer (5), pH 4.7. Grind together 17.65 gm. of $Na_3C_6H_5O_7\cdot 2H_2O$ and 8.4 gm. of $C_6H_8O_7\cdot H_2O$ to a fine powder.

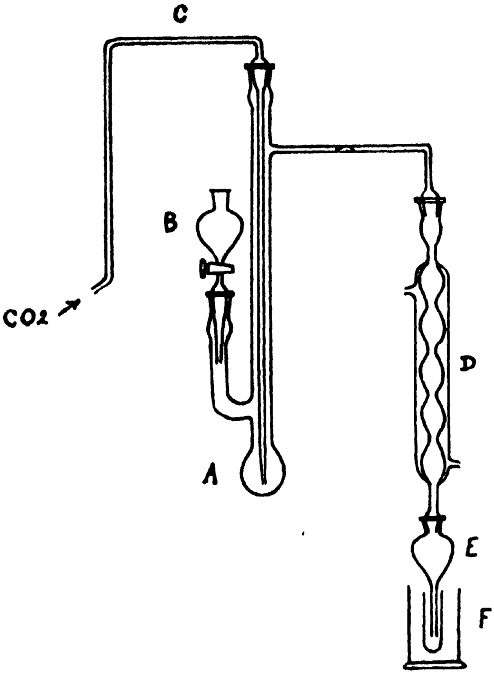


Fig. 1. Distillation apparatus

2. A solution of freshly crystallized *peri*-naphthindan-2,3,4-trione hydrate; 0.1 gm. per 100 cc. of distilled water.

3. α-Amino acid solutions of the concentrations stated in Table I.

4. Fabinyi's reagent. (*a*) 16 cc. of freshly distilled salicylaldehyde made up to 250 cc. with absolute alcohol. (*b*) Exactly 10.5 N sodium hydroxide solution.

Purification of peri-Naphthindan-2,3,4-trione Hydrate—The compound (prepared by Errera (11)) is dissolved in a minimum amount of hot water and ascorbic acid is added to complete the precipitation of dihydroxy-*peri*-naphthindenone (*cf.* Moubasher (12)). The precipitate is filtered off, washed with a few cc. of hot water, suspended in a few cc. of distilled water, and treated with a saturated solution of bromine water. The color of the substance fades to a light yellow. Sufficient distilled water is then added and the suspension is boiled until the solid dissolves. The solution is filtered while hot, and the filtrate is concentrated in the presence of 2 drops of bromine water and left to cool. Almost colorless needles are obtained, m.p. 270°.

Procedure

50 mg. of the solid buffer are placed in the flask (*A*). 1 cc. of the α-amino acid solution and 1 cc. of the *peri*-naphthindan-2,3,4-trione hydrate solution are added. Carbon dioxide is allowed to pass through for about 2 minutes and then heating is carried out until half of the solution distills off. The color of the solution is orange at first, changing to red and lastly pink. Distilled water (4 cc.) is added during the distillation through the separatory funnel (*B*) in order to transfer the last traces of the aldehyde formed to the receiver.

TABLE I

Estimation of Amino Acids with peri-Naphthindan-2,3,4-trione Hydrate

Amino acid	Amount of amino acid used	Equivalent quantity of corresponding aldehydes	Amount of distilled aldehyde solution	Reading of distilled aldehyde solution (R_2)	Concentration of standard aldehyde (C_1)	Reading of standard aldehyde solution (R_1)	Concentration of distilled aldehyde solution (C_2)	Concentration in 10 cc.
	γ	γ	cc.		γ per cc.		γ	γ
Valine	240	147	2	38.8	37	31.5	30	150
Leucine	250	163.8	4	20	54.2	23	62.3	155.7
Phenylaminoacetic acid	200	154.6	3	32.0	41.6	35.4	46	153.3
Aspartic acid	200	66	3	34.6	41	17.2	20.4	68
Alanine	200	98	3	35.9	41	24.5	27.9	93

The distillate is diluted to 10 cc. with distilled water. A known amount (*cf.* Table I) of this solution is treated with Fabinyi's reagent (2 cc. of sodium hydroxide solution and 1 cc. of salicylaldehyde solution). The mixture is placed in a water bath at 50° for 70 minutes, the orange-red color is developed, and the solution is cooled for 10 minutes and read against a solution of known concentration of the corresponding aldehyde treated with Fabinyi's reagent under the same conditions. The color is stable for 24 hours. The reading is carried out with the Universal colorimeter.

By the application of the equation, $R_1/R_2 \times C_1 = C_2$, the results recorded in Table I were obtained. R_1 = reading of the standard aldehyde solution; R_2 = reading of the distilled aldehyde solution; C_1 = concentration of the standard aldehyde solution; C_2 = concentration of the distilled aldehyde solution resulting from the equation.

SUMMARY

1. *peri*-Naphthindan-2,3,4-trione hydrate is used in the microestimation of the following α-amino acids: alanine, aspartic acid, valine, leucine, and phenylaminoacetic acid.

2. Fabinyi's reaction is given by acetaldehyde, isovaleraldehyde, and benzaldehyde and these aldehydes may be estimated colorimetrically.

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DIRECT UTILIZATION OF MALTOSE BY ESCHERICHIA COLI*

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In the course of genetic studies, a mutant strain of *Escherichia coli* was developed which is characterized by rapid fermentation and oxidation of maltose but not of glucose. Since this mutant offered an excellent opportunity to investigate the so called direct utilization of disaccharides (1), a study of the enzyme systems involved in maltose decomposition was undertaken.

Experiments with dry cell preparations from the mutant strain led to the conclusion that maltose is initially transformed to polysaccharide and glucose. This reaction is followed by the phosphorolytic decomposition of the polysaccharide and the usual sequence of glycolytic steps. While the work was in progress, Monod and Torriani reported the discovery of a polysaccharide-forming enzyme in *E. coli* which they called "amylomaltase" and described a method of separating it from other enzymes involved in fermentation (2). They showed that neither phosphate nor glucose-1-phosphate is involved in the transformation of maltose to polysaccharide and that the enzyme is adaptive in nature, being formed only when maltose is used as substrate for the bacteria. They represent the action of "amylomaltase" on maltose by the following equation:

$$n \text{ maltose} \rightarrow n \text{ glucose} + [\text{glucose}]_n$$

Monod and Torriani further found that, if the glucose is removed as it is formed in the reaction, the polysaccharide produced is of the "starch" type, giving a blue complex with iodine. If, on the other hand, the glucose is allowed to accumulate, no product giving a blue color with iodine appears.

The work presented in this paper deals with the integration of the action of amylomaltase with other metabolic processes of the cell. We have demonstrated that the reaction catalyzed by amylomaltase is reversible

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and that the polysaccharide formed is phosphorolytically decomposed to glucose-1-phosphate. The reversibility of the reaction probably accounts for the low molecular weight of the polysaccharide formed in the presence of glucose. By taking advantage of this observation, short chain reducing dextrins have been produced from maltose, and maltose itself has been synthesized from glucose and glucose-1-phosphate.[1]

Amylomaltase appears to belong to the same group of enzymes for which we have proposed the name "transglycosidases" (3) and which includes the dextran- and levan-forming enzymes of certain bacteria and the sucrose phosphorylase of *Pseudomonas saccharophila*. It is probable that this type of enzyme plays an important rôle in the biological decomposition and synthesis of various carbohydrates.

Materials and Methods

The bacteria used in these experiments are all derived from *Escherichia coli* strain K-12. Strain Y-10 is a nutritional mutant requiring threonine, leucine, and thiamine, but fermentatively normal. Strain W-108 was isolated from populations of Y-10 treated with ultraviolet light on the indicator medium, eosin-methylene blue-lactose agar. This mutant is substantially unable to ferment lactose, glucose, or maltose. When large populations of W-108 were inoculated into a maltose synthetic medium, secondary mutations restoring the capacity to ferment this sugar were selected. For the most part, they proved to be reverse mutations to the Y-10 type, but one strain, W-327, was isolated which remained lactose- and glucose-negative, although maltose-positive. Genetic tests on W-327 showed that its ability to utilize maltose was derived from a mutation at a locus different from that involved in the mutation from Y-10 to W-108 (4).

The bacteria (strain W-327) were grown on a rotary shaker in a medium containing 1 per cent peptone, 0.3 per cent beef extract, 0.5 per cent NaCl, and 0.5 per cent maltose. Dry cell preparations were made according to the method of Lipmann (5), the bacteria being harvested after 12 to 18 hours of growth and dried *in vacuo* over P_2O_5. For enzymatic studies, suspensions of the dry cells were usually made in 0.1 M $NaHCO_3$ saturated with CO_2 and incubated in an atmosphere of CO_2 at 30°. In the present work, no attempts were made to separate or purify the various enzymes involved in maltose metabolism.

Determinations of reducing sugar were carried out with the method of Hassid (6). Glucose was estimated from the decrease in reducing value after fermentation with *Torula monosa*. Maltose was determined from the difference between reducing values obtained after fermenting first with suspensions of *T. monosa* and then with *Saccharomyces cerevisiae.* For these tests *S. cerevisiae* was grown on yeast extract-maltose agar. Since there is some evidence that low molecular weight dextrins may be slowly attacked by *S. cerevisiae,* the fermentation was not allowed to proceed for more than 20 minutes after the rapid evolution of CO_2 had ceased. Even under these conditions, it is probable that the values for maltose when present together with dextrins may have been too high.

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[1] Since the preparation of this manuscript, Torriani and Monod have shown the reversible nature of the amylomaltase reaction (14).

Phosphoric esters of sugars were identified and estimated quantitatively by the methods described by Umbreit *et al.* (7).

EXPERIMENTAL

Utilization of Maltose by Intact Resting Cells

The rates of oxidation and fermentation of maltose and glucose respectively were determined with resting cell suspensions in a Warburg respirometer. The bacteria were grown in yeast-maltose medium, and washed and suspended in 0.03 M phosphate buffer at pH 6.8 for experiments on respiration and in 0.1 M $NaHCO_3$ for studies on fermentation. Oxygen uptake was determined in air, while fermentation was measured as CO_2 production from bicarbonate in an atmosphere of CO_2.

In a typical experiment the following rates were observed:

	Fermentation	Oxygen uptake
Endogenous	3 c.mm. CO_2 per 5 min.	4 c.mm. per 5 min.
0.025 M glucose	4 " " " 5 "	7 " " 5 "
0.0125 " maltose	26 " " " 5 "	17 " " 5 "

To determine whether the entire maltose molecule is fermented, a heavy suspension of bacteria was allowed to ferment the sugar in a bicarbonate-phosphate mixture in an atmosphere of CO_2. Determinations of residual maltose and tests for glucose were made periodically. The rate of maltose disappearance was found to be quite constant and no appreciable quantity of glucose could be detected at any time in the medium. The results can be summarized as follows in mg. per ml.: maltose initially present, 3.6; maltose after 45 minutes at 30°, 2.4; maltose after 90 minutes at 30°, 1.2; maltose after 135 minutes at 30°, 0.1; maltose after 180 minutes at 30°, 0.0; and glucose, less than 0.1 mg. at any time.

Maltose Fermentation with Dry Cell Preparations

Dry cell preparations were found to carry out a vigorous fermentation of maltose. They also showed varying rates of endogenous fermentation, which presumably involves the decomposition of reserve carbohydrate. The rate of glucose fermentation was not appreciably higher than the endogenous rate. Fermentation was almost completely inhibited when either fluoride or iodoacetate was added. Since glucose-1-phosphate was thought to be a probable intermediate in the decomposition of maltose, the rate of fermentation of this compound alone and together with glucose was also tested. The observed high rate of fermentation of glucose-1-phosphate was in agreement with the hypothesis that this phosphoric ester is involved in maltose fermentation. The results are shown in Table I. Glucose accumulated in the medium during the fermentation of maltose by dry cell preparations, a phenomenon which was not observed with intact cells.

TABLE I

Fermentation of Sugars by Dry Cell Preparations of E. coli W-327

1.4 ml. of 3 per cent dry cell preparation in 0.1 M bicarbonate, 0.01 M phosphate, at pH 6.8, incubated in an atmosphere of CO_2 at 30°. Experiments 1 and 2 were performed with different batches of dry cells.

Experiment No.	Substrate	Other additions	CO_2 evolved from bicarbonate per 5 min.
			c.mm.
1	None		8
	0.05 M glucose		11
	0.025 " maltose		49
	0.025 " "	0.001 M iodoacetate	2
	0.025 " "	0.05 " fluoride	6
2	None		2
	0.05 M glucose		5
	0.025 " maltose		31
	0.025 " glucose + 0.025 M glucose-1-phosphate		31
	0.05 M glucose-1-phosphate		50

In unpublished studies with *Lactobacillus bulgaricus* it had previously been shown that glucose was not fermented rapidly by dry cell preparations of bacteria grown with lactose as substrate. However, the addition of a small amount of lactose or galactose caused not only the fermentation of these sugars, but also a subsequent rapid fermentation of glucose. To learn whether a similar situation occurs in the fermentation of maltose by preparations of strain W-327, minute amounts of maltose were added together with a large quantity of glucose. In all cases, rapid fermentation was observed only for a short time and the amount of CO_2 evolved was proportional to the amount of maltose added. This indicates that maltose does not act as a "starter" for glucose utilization by such dried preparations.

It could be shown that phosphate is esterified during the fermentation of maltose. However, in the absence of metabolic inhibitors, the uptake of orthophosphate was not very great, and usually somewhat irregular. In the absence of substrate or in the presence of glucose, phosphate was also esterified but at a considerably lower rate. When fluoride, and especially when iodoacetate, was added together with maltose to the bacterial preparations, a very significant uptake of orthophosphate was observed. Under similar conditions, little or no esterification occurred without substrate or with glucose (see Table II).

In experiments in which glucose-1-phosphate was added to the dry cells it was found that this compound disappeared very rapidly, while glucose-6-phosphate, fructose-6-phosphate, and a polysaccharide which gave a brown to blue color with iodine appeared in the medium. This indicates that phosphoglucomutase, phosphohexoisomerase, and a phosphorylase similar to those found in muscle and potato are present in the bacteria. It is known that fluoride interferes with the enzyme phosphoglucomutase which converts glucose-1-phosphate into glucose-6-phosphate. It was therefore expected that the addition of fluoride, but not of iodoacetate, would decrease the rate of conversion of glucose-1-phosphate to other esters, and consequently increase polysaccharide formation. This was found to be the case with the dry cell preparations.

Polysaccharide Formation from Maltose

When dry cell preparations treated with iodoacetate to prevent fermentation were allowed to act on maltose in the absence of added phosphate, glucose and polysaccharide were produced rapidly. In some experiments, approximately 1 mole of glucose was formed per mole of maltose decomposed. This is in agreement with the postulated equation for amylomaltase action. In most experiments, however, the ratio was found to be somewhat less than unity, but in no case less than 0.76. It is possible that errors in the determination of maltose and of polysaccharide, some sources of which have already been mentioned, may have contributed to the low ratios. However, a perfectly reasonable explanation for such results lies in the nature of the polysaccharide produced in the presence of glucose. This will be discussed later in the paper.

The polysaccharide produced from maltose did not give a blue color with iodine. This was in agreement with Monod's observation that iodine-colored polysaccharide is not formed if glucose is allowed to accumulate during the reaction. The following experiment was carried out to elucidate the nature of the polysaccharide produced under these conditions. 40 ml. of a mixture containing approximately 3 per cent dry cells, 0.1 M $NaHCO_3$, 0.002 M sodium iodoacetate, and 0.2 M maltose were incubated at 30° for 180 minutes in an atmosphere of CO_2. The reaction was stopped by the addition of trichloroacetic acid to a concentration of 6 per cent. The precipitate was extracted with 6 per cent trichloroacetic acid and the supernatants combined. The supernatant solution was passed through ion exchange columns and concentrated by vacuum distillation. It was then analyzed for free glucose, maltose, and total glucose released by acid hydrolysis. The following results were obtained in micromoles per ml.: initial maltose, 192 ± 2; maltose decomposed, 114 ± 3; free glucose produced, 112 ± 2; and glucose as polysaccharide, 116 ± 3.

The major portion of the solution was then fermented with *S. cerevisiae* to remove maltose and glucose, again passed through ion exchange columns, and evaporated to dryness under a vacuum. The remaining material was separated into three fractions on the basis of solubility in alcohol. A minor fraction (No. 1) (100 mg.), soluble in hot absolute alcohol, was syrupy and could not be properly characterized. Fraction 2, soluble in hot 95 per cent alcohol but not in absolute alcohol, and the remaining material, which was insoluble in 95 per cent alcohol but soluble in hot 85 per cent alcohol, contained most of the polysaccharide. 300 mg. were recovered in Fraction 2 and 220 mg. in Fraction 3. Both Fractions 2 and 3 were white solids and consisted almost entirely of short chain reducing dextrins. Neither substance formed an insoluble osazone.

Fraction 2 had a reducing value to alkaline ferricyanide corresponding to 44.7 per cent of an equivalent amount of glucose. Acid hydrolysis of this material yielded 87.5 per cent of the theoretical amount of glucose, while hydrolysis with β-amylase resulted in its breakdown chiefly to maltose (83.7 per cent of the theoretical reducing value). Glucose and maltose were identified by their characteristic osazones. Oxidation with hypoiodite indicated that, on the average, 1 out of 4 glucose units of this dextrin possessed a free carbonyl group. Assuming one reducing group per molecule, the average molecular weight of the substance was found to be 675 by this method. The theoretical value for an unbranched dextrin of 4 glucose units is 664.3. Its specific rotation (*c*, 2 per cent) in water was $[\alpha]_D = +145°$. The average molecular weight of the acetylated material as determined by Niederl and Niederl's modification of Rast's method (8) was found to be 1215 (theoretical for 4 glucose units, 1255).

Fraction 3 had a reducing value corresponding to 42 per cent of an equal amount of glucose and gave yields of 90.7 per cent of the theoretical amount of glucose or 89.4 per cent of maltose when hydrolyzed with acid or with β-amylase respectively. Its specific rotation in water (*c*, 2 per cent) was +162°. Oxidation of the compound with hypoiodite (9) indicated that it consists of 6 or 7 glucose residues having a molecular weight of 1110. The theoretical values for unbranched dextrins of 6 and 7 glucose units respectively are 990 and 1152. The average molecular weight of the acetylated product was found to be approximately 1400, with some decomposition occurring during the determination. The theoretical values for acetylated unbranched dextrins of 5 and 6 glucose units are 1543 and 1831 respectively.

Phosphate Esters Produced in Decomposition of Maltose

In the presence of maltose, added inorganic phosphate was esterified at a rate considerably lower than that of the decomposition of the disaccharide. The phosphorolytic nature of this esterification was indicated by the fact that the addition of fluoride and iodoacetate did not prevent the uptake of phosphate. To determine the nature of the phosphate esters, 20 ml. of a 3 per cent bacterial suspension containing 0.2 M maltose, 0.1 M $NaHCO_3$, 0.083 M Sørensen phosphate buffer at pH 6.8, and 0.002 M sodium iodoacetate were incubated for 240 minutes at 30° in an atmosphere of CO_2. Enzyme action was stopped by the addition of trichloroacetic acid and the phosphate esters precipitated with barium. 93 per cent of the esterified phosphorus was recovered in the barium-soluble fraction and was composed almost exclusively of glucose and fructose monophosphates. The amounts of the three principal products of the reaction, determined by the usual methods and corrected for values obtained in the absence of maltose, were found to be 3.4 μM per ml. of original digest for glucose-1-phosphate, 21.2 for glucose-6-phosphate, and 4.4 for fructose-6-phosphate.

Determinations of reducing values and of the rate of hydrolysis of the esters with acid were in agreement with these values. The formation of the three esters again indicated the presence of a phosphorylase, together with phosphoglucomutase and phosphohexoisomerase in the bacterial preparations. In experiments of short duration in which iodoacetate was employed to inhibit the fermentation of maltose, the addition of fluoride decreased the rate of phosphate uptake slightly but increased greatly the ratio of glucose-1-phosphate to the other esters.

Before Monod's successful separation of amylomaltase, it seemed possible that one or two phosphorolytic enzymes might be involved in the transformation of maltose to polysaccharide. Even after the demonstration that glucose-1-phosphate is not an essential intermediate in this transformation, the mechanism of the formation of the phosphate esters remained to be elucidated. Maltose, itself, or the polysaccharide, or both compounds might undergo phosphorolytic cleavage by one or more enzymes. Several lines of evidence, however, indicate that phosphorolysis is involved not in the primary decomposition of maltose but only in the decomposition of the polysaccharide produced from maltose. Briefly summarized, this evidence consists of the following observations.

1. The rate of breakdown of maltose and of the production of glucose and polysaccharide was not increased by the addition of phosphate to the bacterial preparations (see Table II). Even though a small amount of phosphate was present in the dry cells, one would expect a marked effect

of added phosphate on the rate of carbohydrate transformation if glucose-1-phosphate could serve as an intermediate between maltose and polysaccharide. If phosphorolysis of maltose were simultaneous with polysaccharide formation but independent of it, the addition of phosphate should increase both maltose decomposition and glucose production greatly.

2. The addition of arsenate did not prevent polysaccharide formation from maltose. Arsenate is known to replace phosphate with both sucrose and starch phosphorylases and to cause the decomposition of the carbohydrate substrates of these enzymes to their hexose components (10, 11). It will be seen from Table II that a slight increase in maltose decomposition and a somewhat greater increase in glucose production were observed when arsenate was added to the enzyme preparation, together with maltose. These results support the view that arsenate participates directly only in the decomposition of polysaccharide. Maltose decomposition is presumably affected indirectly, since the removal of the polysaccharide can be expected to increase the extent of maltose decomposition.

TABLE II

Maltose Decomposition with Dry Cell Preparation

3 per cent bacterial preparation in 0.1 M $NaHCO_3$, 0.002 M iodoacetate, incubated at 30° for 90 minutes in an atmosphere of CO_2. Maltose added to Tubes 2, 4, and 5; phosphate added to Tubes 3 and 4; arsenate to Tube 5.

	Micromoles per ml.				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
(*a*) Initial phosphate	2	2	76	76	2
(*b*) " arsenate	0	0	0	0	67
(*c*) " maltose		76	0	76	76
(*d*) Phosphate esterified	0	1	0	18	*
(*e*) Glucose formed	0	47	0	45	65
(*f*) Maltose decomposed	0	55†	0	51†	61†
(*g*) Glucose units as polysaccharide (2(*f*) − (*d* + *e*))	0	62‡	0	39‡	57‡

* Not determined.

† The figures for maltose utilization may be somewhat high, for reasons explained earlier in the text.

‡ Since polysaccharide was determined by difference, the values given may be too high, the error in polysaccharide determination being double the error inherent in the maltose determination.

3. Phosphate esterification with maltose was found to be partially or completely inhibited by the addition of saliva (α-amylase) to the bacterial preparations. Control experiments showed that traces of phosphatase, which may occur in saliva, could not account for this inhibition. Since

polysaccharide but not maltose is decomposed with saliva, this observation supports the conclusion that maltose is not phosphorolyzed.

4. The initial rate of phosphate esterification was found to be the same regardless of whether phosphate was added together with maltose or after the enzyme preparation had been allowed to decompose most of the maltose to polysaccharide and glucose. For instance, a 3 per cent bacterial preparation was found to esterify 6.5 μM of phosphate per ml. in the first 30 minutes after the addition of 100 μM of maltose per ml. Under similar conditions only 4.2 μM were esterified if the initial concentration of maltose was reduced to 50 μM per ml. However, when the phosphate was added 120 minutes after the addition of 100 μM of maltose, the phosphate uptake was found to be 6.6 μM in 30 minutes. At this time, less than 40 μM of maltose remained in the mixture.

Indirect Synthesis of Maltose from Glucose and Glucose-1-phosphate

It seemed very likely that the reaction catalyzed by amylomaltase would be reversible in nature, as are the phosphorolytic reactions involved in the breakdown of starch and sucrose. The occurrence of a reverse reaction would explain Monod's observation that the polysaccharide produced from maltose in the presence of glucose does not form a blue complex with iodine, since long polysaccharide chains would be broken down to dextrins of relatively low molecular weight, in accordance with the equation

$$(C_6H_{10}O_5)_n + m\ C_6H_{12}O_6 \rightarrow (C_6H_{10}O_5)_{n-m} + m\ C_{12}H_{22}O_{11}$$

The reversibility of both maltose decomposition and the phosphorolytic reaction of the mutant strain was clearly demonstrated by the following observations.

1. The addition of glucose, together with glucose-1-phosphate, prevented the formation of a polysaccharide giving a blue complex with iodine. That this was not due to a simple inhibition of phosphorylase activity was shown by the fact that glucose had only a slight inhibitory effect on the nonhydrolytic deesterification of glucose-1-phosphate. It is of interest to record that D-xylose and, to a slight extent, D-mannose also inhibited the formation of the starch-like polysaccharide. D-Fructose, D-galactose, D-arabinose, and L-arabinose had no significant effect. It seems possible that D-xylose and perhaps D-mannose may react with the polysaccharide in the presence of amylomaltase to yield disaccharides analogous to maltose.

2. The addition of glucose to bacterial preparations which had synthesized polysaccharide from glucose-1-phosphate caused the rapid transformation of the polysaccharide to a form which no longer gave a blue-colored complex with iodine. The experiment was conducted as follows: A 3 per cent dry cell preparation was incubated with 0.2 M glucose-1-

phosphate at pH 6.8 for 30 minutes at 30°. This mixture was then boiled and incubated with an equal volume of 6 per cent active dry cell preparation in the presence and in the absence of 0.25 M glucose. Before incubation the mixture gave a deep brown color with iodine which changed quickly to blue on standing. In the absence of glucose, the material still gave a dark brown color with iodine after 30 minutes of incubation and a reddish brown color after 60 minutes. This slow color change is due either to a slow hydrolytic cleavage of the polysaccharide by bacterial amylase or to the phosphorolytic decomposition due to the gradual conversion of glucose-1-phosphate to other esters. In the presence of glucose, on the other hand, the color with iodine changed to pale reddish brown after 7 minutes and disappeared after 10 minutes of incubation.

TABLE III

Synthesis of Maltose and Polysaccharide with Dry Cell Preparation

3 per cent bacterial preparation in 0.1 M $NaHCO_3$, 0.002 M iodoacetate, 0.05 M NaF, incubated for 30 minutes at 30° in an atmosphere of CO_2. Glucose added to Tubes 2 and 3; glucose-1-phosphate added to Tubes 1 and 3 (solution adjusted to pH 6.8). About 2 μM of inorganic phosphate per ml. of preparation were present initially.

	Micromoles per ml.		
	Tube 1	Tube 2	Tube 3
Initial glucose	0	174	174
" glucose-1-phosphate	75	0	75
Total disappearance of glucose-1-phosphate*	63	0	52
Inorganic phosphate produced	49 ± 1	0	49 ± 1
Disappearance of free glucose†	−2 ± 1	2 ± 3	20 ± 3
Maltose formed	0	0	17 ± 1
Color with iodine	Blue	None	None

* Disappearance of ester due to transformation to other phosphate esters, formation of polysaccharide, and of maltose.

† Slight production of glucose from glucose-1-phosphate evident in Tube 1.

3. When glucose was added together with glucose-1-phosphate to the bacterial preparations, the production of maltose and reducing dextrins was observed. As in the previous experiments, maltose was estimated as sugar fermentable with *S. cerevisiae* but not with *T. monosa*. In addition, it was identified by the microscopic examination of its osazone. Maltose was not formed when glucose-1-phosphate alone or glucose alone was added to the enzyme preparations (see Table III).

Experiments with Wild Type Parent Strain

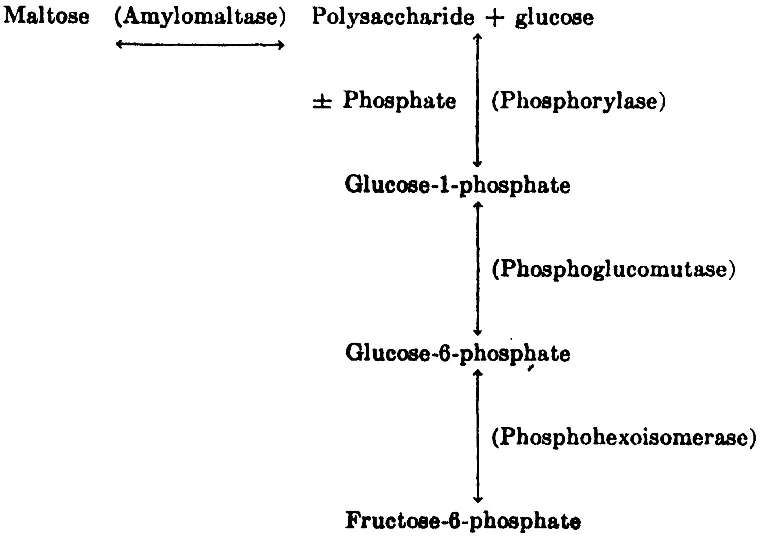
It was of interest to check whether the enzymes involved in the metabolism of maltose by the mutant strain W-327 are also present in the wild type strain K-12, from which the mutant had been derived indirectly. For this purpose, a dry cell preparation of *E. coli* K-12 was made from a culture grown with maltose. This preparation caused an esterification of inorganic phosphate when maltose was added together with iodoacetate. No phosphate was taken up by iodoacetate-treated cells in the absence of substrate or in the presence of glucose. A polysaccharide giving a blue color with iodine was formed when the preparation was allowed to act on glucose-1-phosphate in the presence of iodoacetate and fluoride. The addition of glucose prevented the production of the iodine-colored compound.

These observations were accepted as evidence that the wild type strain adapted to maltose does possess the same enzymes as the mutant strain.

Strain W-327 presumably differs from strain W-108, from which it was obtained directly, in the restoration of the original wild type metabolism of maltose but not of glucose or lactose.

DISCUSSION

From the above experiments, it seems reasonable to conclude that the initial stages of the fermentation of maltose by dry cell preparations of *E. coli* W-327 involve the set of reversible reactions shown in the accompanying scheme.



This scheme, however, fails to account for the observation that intact cells produce no glucose in the metabolism of maltose and yet possess practically no ability to ferment or oxidize glucose when this sugar is supplied in the medium. The situation is analogous to that found in the oxidation of sucrose, trehalose, and melibiose by *P. saccharophila* (12, 13). When grown with sucrose, this organism possesses a sucrose phosphorylase and an invertase, both of which decompose sucrose with the production of fructose. Yet fructose supplied in the medium remains practically unattacked by the intact cells, while the entire sucrose molecule is rapidly metabolized. Bacteria grown in the presence of trehalose possess an active trehalase which hydrolyzes the disaccharide to glucose. No evidence for either a phosphorylase or a special kinase could be found in experiments with dry cell preparations and intact cells. Yet adapted intact cells are capable of oxidizing trehalose at a much greater rate than glucose. Similarly, bacteria grown with raffinose or melibiose as substrate can oxidize melibiose at a much greater rate than the constituent monosaccharides glucose and galactose, provided that all these sugars are supplied in high concentration. As with trehalose, only a hydrolytic cleavage of melibiose appears to take place. In all of the above cases, the demonstration of enzymes responsible for the primary decomposition of disaccharides fails to explain the discrepancy in the rates utilization by intact cells of the disaccharides on the one hand and of hexoses on the other.

If the proposed course of maltose breakdown by *E. coli* W-327 is accepted, an explanation must be sought for the feeble metabolism of glucose by this strain. Although no study of hexokinase activity in either the mutant or the wild type strain has as yet been made, it seems unlikely that the absence of this enzyme would account for the difference between the mutant and parent cultures. If hexokinase were lacking, glucose should accumulate in the medium during the metabolism of maltose. This is not the case. A different impairment of the phosphate metabolism of the organism might offer a possible explanation for the anomalous behavior of the mutant.

Regardless of the nature of the difference between the parent and mutant strain of *E. coli*, it seems clear that amylomaltase plays an important rôle in the metabolism of this species. It is of particular interest that this enzyme is similar to the "sucrose phosphorylase" of *P. saccharophila*, and to the bacterial enzymes responsible for the formation of dextran and levan from sucrose, in that all of these enzymes catalyze the exchange of glycosidic linkages (3). Unlike levan production, the formation of polysaccharide from maltose is readily reversible. This is probably due largely to a lower energy content of the glycosidic bond in maltose than in sucrose.

In addition, it seems likely that the rate of the reverse reaction is greater with the polysaccharide of *E. coli* than with levan because the latter compound has a very high molecular weight and is therefore normally present in very low molar concentrations.

Many important details of the mechanism of polysaccharide formation from maltose remain to be elucidated through studies with purified amylomaltase. The experiments described to date do not show whether a polysaccharide nucleus is necessary for the initiation of polysaccharide synthesis. In comparable reactions catalyzed by muscle and potato phosphorylases it is known that catalytic amounts of polysaccharide are required. If no "starter" is needed, the initial reaction would involve 2 molecules of maltose which would be converted to 1 molecule of trisaccharide and 1 of glucose. It has been found experimentally that less than 1 mole of glucose is usually formed for each mole of maltose decomposed. This was to be expected, since the polysaccharide produced from maltose was found to be of low molecular weight. For instance, in the formation of a reducing tetrasaccharide possessing the formula $(C_6H_{10}O_5)_3$-$C_6H_{12}O_6$, only 2 moles of glucose should be evolved from 3 moles of maltose, in accordance with the equation $3C_{12}H_{22}O_{11} \rightarrow (C_6H_{10}O_5)_3C_6H_{12}O_6 + 2C_6H_{12}O_6$. Another important question which has not been answered so far is whether catalysis by amylomaltase is limited to a transformation of disaccharide to polysaccharide or whether it also involves the condensation of polysaccharide molecules such as the following.

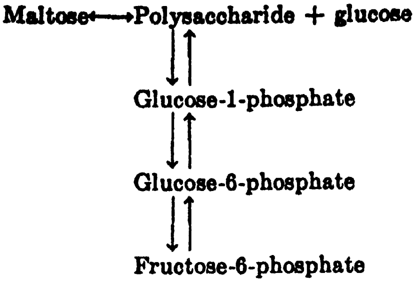
$$(C_6H_{10}O_5)_nC_6H_{12}O_6 + (C_6H_{10}O_5)_mC_6H_{12}O_6 \leftrightarrow (C_6H_{10}O_5)_{n+m}C_6H_{12}O_6 + C_6H_{12}O_6$$

Finally, the bearing of these studies on gene action may be mentioned. As far as they go, these experiments suggest that a single gene mutation, as in W-108, may interfere with the action or formation of several enzymes: in this case lactase, amylomaltase, and possibly hexokinase. It cannot yet be said whether these are primary or secondary effects of the mutation. On the other hand, a "suppressor" mutation, as in W-327, may undo part of these effects, restoring the amylomaltase function.

SUMMARY

1. A mutant of *Escherichia coli* was found capable of carrying out rapid oxidation and fermentation of maltose but not of glucose.

2. Studies with dry cell preparations indicated that both the mutant and the wild type parent strains contain the enzymes amylomaltase, phosphorylase, phosphoglucomutase, and phosphohexoisomerase. The first steps in maltose decomposition could be postulated as shown in the accompanying scheme.



3. When glucose is allowed to accumulate during the decomposition of maltose, the polysaccharide produced by amylomaltase was found to consist of reducing dextrins composed, on the average, of from 4 to 6 glucose units.

4. Due to the reversible nature of the above reactions, maltose and reducing dextrins were produced when glucose-1-phosphate and glucose were added together to the preparations. In the absence of glucose, only a starch-like polysaccharide was formed from glucose-1-phosphate.

5. The proposed mechanism for a "direct" utilization of maltose fails to explain the impaired ability of the mutant to utilize glucose.

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THE UTILIZATION OF D-GLUTAMIC ACID BY LACTOBACILLUS ARABINOSUS 17-5*

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L-Glutamic acid was first determined microbiologically with *Lactobacillus arabinosus* 17-5 by Kuiken *et al.* (2) in 1943. It was reported that D-glumatic acid was inactive for *L. arabinosus*, since 20 γ of L-glutamic acid gave the same growth response as 40 γ of DL-glutamic acid. The following year, Dunn *et al.* (3) found that L-glutamic acid could be determined accurately with *L. arabinosus*, but only in the absence of D-glutamic acid, since, over the range, 1.5 to 4.0 mg. per cent, DL-glutamic acid was markedly more active for this microorganism than L-glutamic acid. These results were confirmed in 1945 by Baumgarten *et al.* (4), who showed, in addition, that D-glutamic acid (in the absence of L-glutamic acid) promoted less growth than an equal amount of L-glutamic acid (in the absence of D-glutamic acid). Other workers (5–7) have reported activities of 51 to 75 per cent[1] for DL-glutamic acid and of 7 to 11 per cent for D-glutamic acid. It is evident, therefore, that the activity of D-glutamic acid for *L. arabinosus* 17-5 varies markedly under different conditions.

Recently Lyman and Kuiken (8) showed that the utilization of D-isoleucine, but not D-glutamic acid, by *L. arabinosus* is influenced by the form of vitamin B_6 in the basal medium. The present report is concerned with the effect of L-aspartic acid, D-aspartic acid, and natural asparagine on the utilization of D-glutamic acid by *L. arabinosus* 17-5.

EXPERIMENTAL

The basal medium was that described by Dunn *et al.* (9). The response of *L. arabinosus* 17-5 to L-, DL-, and D-glutamic acids was measured in the absence and in the presence of added L-aspartic acid, D-aspartic acid, and natural L-asparagine. The three forms of glutamic acid were tested at final concentrations of 2.4 γ to 256 γ per ml., and L-aspartic acid, D-aspartic acid, and asparagine were employed at final concentrations of 400, 800, 1600, and 3200 γ per ml.[2]. The tests were made in 3 ml. volumes in 4

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* Paper 56. For Paper 55, see Dunn *et al.* (1). This work was aided by grants from the American Cancer Society through the Committee on Growth, National Research Council. The authors are indebted to Samuel Eiduson and Ruth B. Malin for technical assistance.

[1] Calculated on weight per cent basis compared with L-glutamic acid.

[2] These concentrations were in addition to the natural asparagine (60 γ per ml.) present in the unaltered basal medium.

inch test-tubes in a manner analogous to that previously described for assays with *L. arabinosus* (9). The incubation period was 3 days. The results are given in Figs. 1 to 3 and in Tables I and II.

DISCUSSION

The curves presented in Fig. 1 and the activities calculated for D- and DL-glutamic acids (Tables I and II) indicate that these forms were more active (up to 28 per cent for the D- form and up to 40 per cent for the DL form) than L-glutamic acid in the unaltered basal medium. It may be seen, however (Fig. 2, Tables I and II), that in the presence of added L- or D-aspartic acid much or all of the activity of the D-glutamic acid was lost. The activity of DL-glutamic acid at low concentrations was reduced by additions of asparagine (Table I). At higher concentrations, under these conditions, the activity of DL-glutamic acid was greater than that of L-glutamic acid, apparently due to a depression of the L-glutamic acid curve, but not of the DL-glutamic acid curve, in the range of concentrations above 40 γ per ml. (Fig. 3). Curves similar to those in Fig. 3 were obtained by Dunn *et al.* (3) and by Baumgarten *et al.* (4), undoubtedly as a result of the relatively high concentrations of asparagine employed in their media.

That added aspartic acid will depress the initial segment of the L-glutamic acid curve obtained with *L. arabinosus* was first noted by Lewis and Olcott in 1945 (5).[3] Similar results with asparagine, as well as aspartic acid, were later noted by Baumgarten *et al.* (4) and Brickson *et al.* (11). This effect on the L-glutamic acid curve is evident, but not pronounced, in the present work. Compare, for example, the L-glutamic acid curves in Figs. 1 to 3.

It has been observed by a number of workers (5–7, 11–13) that the initial lag so often present in the L-glutamic acid curve with *L. arabinosus* is not observed in the corresponding L-glutamine curve. For this reason it has been inferred by some of these authors that L-glutamic acid is converted to glutamine before being utilized by *L. arabinosus*, and that aspartic acid interferes with the formation of glutamine from L-glutamic acid. This hypothesis would seem to be negated, however, by the observations of the present authors[4] and Pollack and Lindner (14) that, under some conditions, L-glutamic acid is more active than glutamine for *L. arabinosus*.[5]

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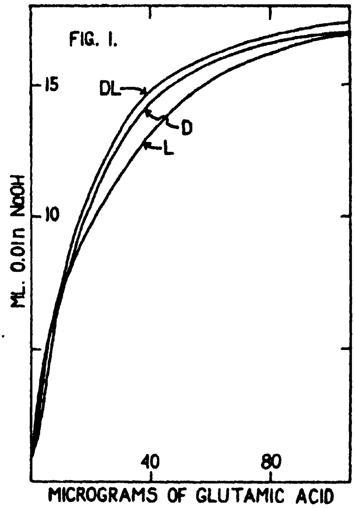
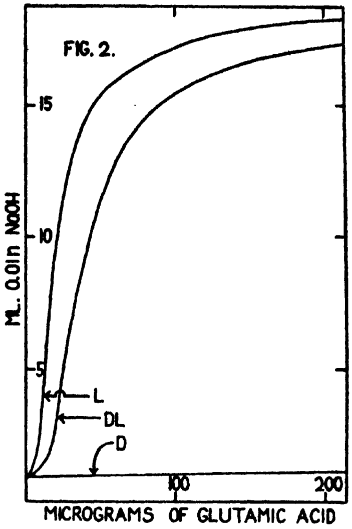
[3] These authors reported that arginine contributed to the inhibition of glutamic acid by aspartic acid.

[4] Unpublished data obtained by M. S. Dunn, S. Shankman, and M. N. Camien.

[5] The results of these experiments would be invalidated if the glutamine samples were very impure. Pollack and Lindner (14) did not report the purity of their glu-





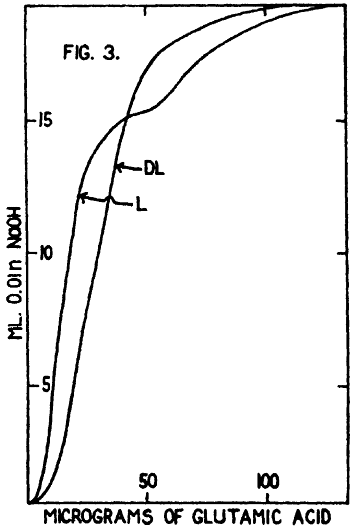


FIG. 1. Response of *L. arabinosus* 17-5 to L-, DL-, and D-glutamic acids in the unaltered basal medium. The micrograms of glutamic acid and the ml. of 0.01 N NaOH are the amounts calculated per ml. of final solution. The L- and DL-glutamic acids were analytically pure products of Amino Acid Manufactures. The D-glutamic acid was the product described previously (10).

FIG. 2. Response of *L. arabinosus* 17-5 to L-, DL-, and D-glutamic acids in the basal medium supplemented with L-aspartic acid at a concentration of 1600 γ per ml. of final solution. The L-aspartic acid was a C.P. product of Amino Acid Manufactures.

FIG. 3. Response of *L. arabinosus* 17-5 to L- and DL-glutamic acids in the basal medium supplemented with natural asparagine (Pfanstiehl product) at a concentration of 3200 γ per ml. of final solution.

TABLE I

Calculated Activity of DL-*Glutamic Acid at Different Dosage Levels and in Different Media*

Test level	Titration*	Activity†	Test level	Titration*	Activity†	Test level	Titration*	Activity†
Medium 1			**Medium 2**			**Medium 3**		
γ per ml.		*per cent*	*γ per ml.*		*per cent*	*γ per ml.*		*per cent*
4.8	4.32	83	16	2.60	47	24	3.87	48
8	6.42	106	24	5.75	51	32	7.01	52
16	9.87	131	32	8.40	53	40	8.76	50
24	12.03	138	40	10.40	54	53	11.89	54
40	14.83	140	53	13.32	58	107	15.84	55
Medium 4			**Medium 5**			**Medium 6**		
53	4.08	52	53	0.52	50	16	3.63	57
67	7.95	53	107	8.96	51	24	6.76	57
80	10.36	52	160	13.06	52	32	9.26	56
107	13.77	52	213	14.85	49	40	11.16	56
133	15.55	52				53	13.70	57
Medium 7			**Medium 8**			**Medium 9**		
16	2.24	50	24	2.00	50	24	1.32	50
24	5.58	55	32	3.93	52	32	2.09	44
32	8.10	55	40	6.57	50	40	4.68	47
40	9.92	55	53	9.78	51	53	8.84	52
53	12.10	54	107	16.33	53	107	15.30	53
Medium 10			**Medium 11**			**Medium 12**		
3.3	1.05	50	6.7	1.49	45	13.3	2.12	58
6.7	2.67	66	13.3	4.36	61	20.0	5.31	58
13.3	8.29	86	20.0	9.11	63	26.7	8.08	59
26.7	13.50	149	26.7	13.59	132	33.3	11.16	64
40.0	15.58	127	40.0	15.42	142	40.0	14.33	82

Medium 1 was the unsupplemented basal medium. Media 2 to 5 were supplemented with L-aspartic acid at concentrations in micrograms per ml. of final solution as follows: 2 (400), 3 (800), 4 (1600), 5 (3200). Media 6 to 9 were supplemented in order with the same amounts of D-aspartic acid (C.P. product of Amino Acid Manufactures). Media 10 to 12 were supplemented with natural asparagine as follows: 10 (800), 11 (1600), 12 (3200) micrograms per ml. of final solution.

* Given as ml. of 0.01 N NaOH to titrate 1 ml. of final solution (equivalent to titrations of 10 ml. volumes with 0.1 N NaOH).

† Calculated on weight per cent basis as compared with L-glutamic acid. The values included in the table were selected from similar response ranges as indicated by the corresponding titration values.

That D-glutamic acid may play "some essential rôle in the metabolism of this microorganism" was suggested by Dunn *et al.* (3), because D-glutamic acid (tested as the DL form) was much more active under certain conditions than L-glutamic acid. Although this conclusion may seem to have been unwarranted in the light of some of the present results (Fig. 2, Table II), it is supported by the observation that hydrolysates of *L. arabinosus* cells grown on D-glutamic acid-free media contain relatively large amounts of D-glutamic acid (10, 15).[6]

TABLE II

Calculated Activity of D-Glutamic Acid at Different Dosage Levels and in Different Media

The media were those described in Table I. The activity of D-glutamic acid was not determined for Media 10 to 12, due to insufficient sample.

Test level	Titration*	Activity*	Test level	Titration*	Activity*	Test level	Titration*	Activity*
Medium 1			Medium 2			Medium 3		
γ per ml.		per cent	γ per ml.		per cent	γ per ml.		per cent
6	4.35	67	40	1.17	7.1	53	0.89	4.7
8	5.54	76	107	1.58	5.2	107	1.05	4.2
16	9.13	109	160	1.80	3.8	160	1.34	4.0
32	13.08	125	213	2.67	3.6	213	1.38	3.1
40	14.39	128	267	4.35	3.7	267	1.53	2.7
Medium 4			Medium 5			Medium 6		
53	0.97	0.0	53	0.45	0.0	53	1.44	8.7
107	1.05	0.6	107	0.45	0.0	107	2.37	6.7
160	1.08	0.6	160	0.45	0.0	160	4.67	6.7
213	1.20	1.5	213	0.45	0.0	213	8.12	7.4
			267	0.45	0.0	267	10.93	8.1
Medium 7			Medium 8			Medium 9		
53	1.02	8.1	107	0.81	3.8	53	0.60	0.0
107	1.47	5.9	160	1.02	4.4	107	0.60	0.0
160	2.06	4.8	213	1.17	4.2	160	0.68	3.5
213	3.31	4.7	267	1.35	3.9	213	0.75	3.4
267	5.27	4.7				267	0.94	3.6

* See the corresponding foot-notes to Table I.

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tamine. The sample of glutamine (S. M. A. Corporation product) employed in the unpublished experiments by the present authors was too small for adequate analysis.

[6] Some workers (16, 17) have suggested that D-glutamic acid found in hydrolysates of proteinaceous materials may originate from the racemization of L-glutamic acid. It does not appear likely, however, that nearly complete racemization of

Perhaps a more tenable hypothesis might be that glutamine, D-glutamic acid, and L-glutamic acid are converted to a common intermediate[7] by *L. arabinosus* before being utilized. The observation that neither glutamine nor D-glutamic acid is *consistently* more active than L-glutamic acid may be explained, assuming that the extent of conversion to the intermediate depends on the available compound and the conditions. Studies on the utilization of glutamine by *L. arabinosus* are being carried out to throw further light on this problem.

The results of the present investigation may be of practical application in the microbiological assay of samples containing both enantiomorphs of glutamic acid. It seems unlikely that total glutamic acid could be determined with *L. arabinosus*, since conditions have not been found under which the activities of L- and D-glutamic acids are the same over the entire assay range. It does appear probable, however, that a high degree of specificity for L-glutamic acid in the presence of D-glutamic acid can be induced by adding sufficient aspartic acid to the *L. arabinosus* assay medium. The initial lag in the L-glutamic acid response curve resulting from the increased aspartic acid concentration can be overcome by increasing the incubation time (5), by increasing the amount of inoculum (5, 7), by adjusting the medium to pH 6.0 (7), by adding glutamine to the basal medium (6), or by adding to the basal medium an amount of L-glutamic acid sufficient to initiate growth of *L. arabinosus* under the conditions employed. Although *Streptococcus faecalis* R may also be used to determine L-glutamic acid in the presence of D-glutamic acid (4, 10), *L. arabinosus* has the advantages of more rapid and profuse growth and acid production. The latter microorganism may be preferred for this reason.

It is of interest in this connection that Lewis and Olcott (5) have employed *L. arabinosus* for the determination of L-glutamic acid in the presence of D-glutamic acid. Total glutamic acid was determined by a chemical method (19) and D-glutamic acid was estimated by difference. Although, under the conditions employed by these workers, D-glutamic acid was found to be 7 to 11 per cent as active as L-glutamic acid (which would lead one to expect high results for L-glutamic acid and low results for D-glutamic acid), approximately 10 per cent of the total glutamic acid in edestin and approximately 30 per cent of the total glutamic acid in normal

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L-glutamic acid would take place during the relatively short period of refluxing with acid to which the *L. arabinosus* cells were subjected. Other bacterial products have been shown to yield nearly pure D-glutamic acid on acid hydrolysis (18). Since L-glutamic acid was negligible in the latter hydrolysates, the possibility of the D-glutamic acid having been formed by racemization was excluded.

[7] It does not seem probable that α-ketoglutaric acid or α-hydroxyglutaric acid is an intermediate of this type, since neither is very active in replacing L-glutamic acid for *L. arabinosus* (4, 5).

and carcinomatous tissue from rabbits were estimated to be D-glutamic acid.

SUMMARY

It has been shown that D- and DL-glutamic acids under some conditions are more active than L-glutamic acid in promoting growth of *Lactobacillus arabinosus*, but that increased amounts of aspartic acid in the basal medium partly or completely suppressed the activity of D-glutamic acid for this organism. Asparagine was less effective than aspartic acid in suppressing the activity of D-glutamic acid, particularly at relatively high levels of glutamic acid. It has been suggested that *L. arabinosus* may be used for the determination of L-glutamic acid in the presence of D-glutamic acid if sufficient aspartic acid is added to the basal medium.

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ACTION PATTERN OF CRYSTALLINE MUSCLE PHOSPHORYLASE*

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The question to what extent branched polysaccharides can be degraded by phosphorylase has recently been examined by Meyer and Bernfeld (1), Swanson (2), and Katz, Hassid, and Doudoroff (3). It was found that potato phosphorylase resembled β-amylase in being unable to pass the 1,6 linkage at the branch points. In fact, the terminal chains of either amylopectin or glycogen were removed less completely by potato phosphorylase than by β-amylase (2).

In the case of muscle phosphorylase it was known from experiments with relatively crude preparations that glycogen could be degraded almost completely (4).[1] Swanson (2) found a degradation of about 80 per cent of glycogen or amylopectin in experiments with crystalline muscle phosphorylase. A high concentration of enzyme was used, more enzyme was added at intervals, and the incubation period was quite long. If complete phosphorolysis of glycogen should require another enzyme besides phosphorylase, this enzyme, even though present only in traces as a contaminant of phosphorylase crystals, could have exerted considerable activity under the conditions of Swanson's experiments[2] (*cf.* (5)).

The experiments reported in this paper show that the action of repeatedly recrystallized muscle phosphorylase on branched polysaccharides is similar to that previously reported for potato phosphorylase. Some properties of the limit dextrin formed from glycogen by the action of

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[1] Cori, G. T., unpublished experiments.

[2] A repetition of these experiments by G. T. Cori gave the following results. With a twice crystallized sample of muscle phosphorylase, glycogen was degraded about 80 per cent after prolonged incubation and repeated enzyme additions. On further recrystallizations the apparent property of the enzyme of degrading glycogen beyond the branch points was lost; that is, under the same conditions the degradation did not exceed 46 per cent. The supernatant fluid from the recrystallizations contained a protein which, when added to phosphorylase, resulted again in an almost complete degradation of glycogen. The properties of this factor will be described in a later publication.

muscle phosphorylase are described. This substance was used in an experiment designed to test the theory of the primer effect of polysaccharides.

EXPERIMENTAL

Phosphate was determined by the method of Fiske and Subbarow (6). Glucose-1-phosphate was hydrolyzed completely in 0.1 N HCl for 10 minutes at 100° and determined as free glucose by the method of Nelson (7). A correction for the concurrent hydrolysis of added polysaccharides was applied in all cases; it generally amounted to less than 5 per cent of the reducing value obtained by the hydrolysis of glucose-1-phosphate. No cysteine was added to the reaction mixtures because in the concentration usually used in phosphorylase tests it interferes with the glucose estimations. Since the phosphorylase crystals are suspended in a cysteine buffer of 0.03 M, the cysteine concentration in the reaction mixture was of the order of 0.0006 M or less.

Enzymes—The activity of the enzymes was tested at 30°. Phosphorylase *a* was prepared from rabbit muscle as described by Green and Cori (8), and recrystallized at least three times. The purified enzyme was completely free of amylase activity. Potato phosphorylase was purified by fractionation with ammonium sulfate as described by Hanes (9). Crystalline phosphoglucomutase (10) from rabbit muscle was kindly supplied by Dr. V. Najjar; it contained neither phosphorylase nor amylase. β-Amylase was a maltase-free preparation made from ungerminated wheat by the method of Ballou and Luck (11).

Substrates—Glucose-1-phosphate (1-ester) was prepared according to McCready and Hassid (12) and recrystallized twice; it was free of polysaccharide impurities which prime muscle phosphorylase. Rabbit liver glycogen was used. Specimen 1 ($[\alpha]_D = +200°$; ash 0.25 per cent) was obtained from the Pfanstiehl Chemical Company and Specimen 2 was isolated in this laboratory by the method of Somogyi (13).

LD (glycogen; wheat β-amylase)[3] was prepared by incubating glycogen Specimen 1 (5 per cent) with β-amylase (0.02 per cent) in citrate buffer, pH 6.0, under toluene at 30°. After 16 hours, the hydrolysis calculated in terms of maltose was 47 per cent and had come to a standstill. The residual polysaccharide was precipitated with 2 volumes of methanol, dried, exposed again to the action of the enzyme, and isolated.

LD (glycogen; muscle phosphorylase) was prepared by incubating glycogen Specimen 1 (5 per cent) at 30° with 0.15 M potassium phosphate, pH 7.3, 1×10^{-4} M magnesium chloride, 1.6×10^{-5} M muscle adenylic acid, and 1.5×10^{-2} M cysteine in the presence of 10 mg. per cent of phosphorylase and 2.5 mg. per cent of phosphoglucomutase. After 1 hour, the disappearance of inorganic phosphate corresponded to about 31 per cent degradation and the reaction had come to a standstill. The residual polysaccharide was precipitated by adding 2 volumes of methanol, and dried. A sample was retained for further examination and the remainder was treated again with the two enzymes and isolated. The dry powder was taken up in water, and a small amount of insoluble material was removed by centrifugation. The solution was passed through an Amberlite IR-4-100 anion exchange column and the polysaccharide precipitated with 2 volumes of methanol. The yield corresponded to 54 per cent of the original glycogen.

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[3] Limit dextrins derived from a parent carbohydrate by the exhaustive treatment with a specific enzyme are designated as LD, information which characterizes them being supplied in parentheses.

The material formed an opalescent solution in water at room temperature, was non-dialyzable through a cellophane membrane, gave a much less intense color with iodine than did glycogen, was resistant to hot 10 per cent potassium hydroxide, and was non-reducing to alkaline copper reagents. The limit dextrin was hydrolyzed by salivary amylase and partially hydrolyzed by wheat β-amylase.

Other materials used were kindly supplied by the following investigators: corn amylopectin (Fraction B) and amylose (Fraction A) by Dr. T. Schoch, tuber and cereal starch amylopectins and their wheat β-amylase limit dextrins by Dr. J. E. Hodge, amylopectin formed by *Neisseria perflava* from sucrose by Dr. E. J. Hehre, crystalline α-Schardinger dextrin by Dr. C. S. Hudson, dextran formed by *Leuconostoc mesenteroides* from sucrose, and products of partial hydrolysis of dextran by acid by Dr. A. Jeanes.

A mixture of dextrinic acid homologues (amyloses of 6 or less glucose residues with the reducing group oxidized to —COOH) was obtained in aqueous solution from α-Schardinger dextrin as follows: 35 mg. of the dextrin were dissolved in 0.5 ml. of 7.5 N hydrochloric acid. The solution was kept at 30° for 4 hours, neutralized, and brought to a volume of 1.2 ml. The degree of hydrolysis (calculated as glucose) amounted to 27 per cent. To 0.5 ml. of the neutralized hydrolysate, 0.1 ml. of 0.1 N iodine solution and 1.5 ml. of 0.1 N sodium hydroxide were added, and the mixture was kept in the dark for 20 minutes at room temperature. The solution was acidified, extracted repeatedly with toluene, and neutralized. When the solution was cooled, some residual dextrin containing adsorbed iodine precipitated and was removed. The filtrate containing the dextrinic acids was colorless and free of iodine. Copper reduction tests showed that the reducing groups had been oxidized to an extent of more than 96 per cent.

Results

Phosphorolysis of Polysaccharides. Glycogen and Amylopectin—The course of phosphorolysis at two enzyme and glycogen concentrations is plotted in Fig. 1. The limit of phosphorolysis varies somewhat for the two glycogen specimens investigated, but it is evident that in both instances a major fraction of the linkages resists cleavage. At pH 7.3 the reaction would reach equilibrium when the ratio, inorganic P:1-ester P, is about 3. In the experiments in Fig. 1 the final ratios were 20 to 40 and the slowing down of the reaction cannot therefore be explained by an approach to equilibrium. There was the possibility that the slowing down was caused by an inactivation of the enzyme, but control experiments showed that this was not the case. Aliquots of the reaction mixture were removed after varying lengths of time of incubation, mixed with an equal

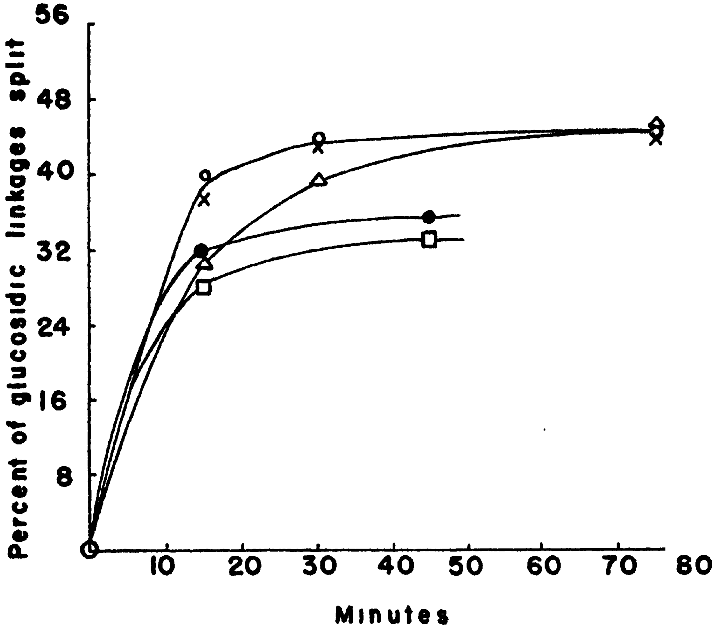

FIG. 1. Phosphorolysis of glycogen with an excess of inorganic phosphate. The reaction mixture contained 0.1 M potassium phosphate of pH 7.3 and 1.6 x 10^{-4} M muscle adenylic acid. Muscle phosphorylase protein was dissolved by mixing a crystal suspension with 1 volume of 0.5 M sodium bicarbonate, followed by 10 volumes of water. Glycogen Specimen 1 was used in Experiment A and Specimen 2 in experiment B. Relative enzyme dilutions and initial concentrations of glycogen (as moles of glucose per liter) were as follows: Experiment A, □ 1:50, 0.012; ● 1:50, 0.006; Experiment B, △ 1:100, 0.012; × 1:100, 0.006; ○ 1:50, 0.006. The amount of glucose-1-phosphate formed was determined as free glucose after short acid hydrolysis as described under the methods.

volume of 1.2 per cent glycogen, incubated for 10 minutes, and analyzed for the amount of 1-ester formed. There was no significant loss of phosphorylase activity during 2 hours of incubation.

In the experiment in Fig. 2 the initial phosphate concentration was only slightly greater than that of glycogen. Phosphoglucomutase was added in order to prevent an accumulation of glucose-1-phosphate. Cysteine was present in 0.007 M concentration. The effectiveness of the mutase action was checked at the end of the experiment; only 7 per cent of the total ester phosphate present was easily hydrolyzable in acid. The limit of degradation was 36 per cent and corresponded closely to the limit observed when the same specimen of glycogen was phosphorolyzed in the presence of a large excess of phosphate and in the absence of mutase.

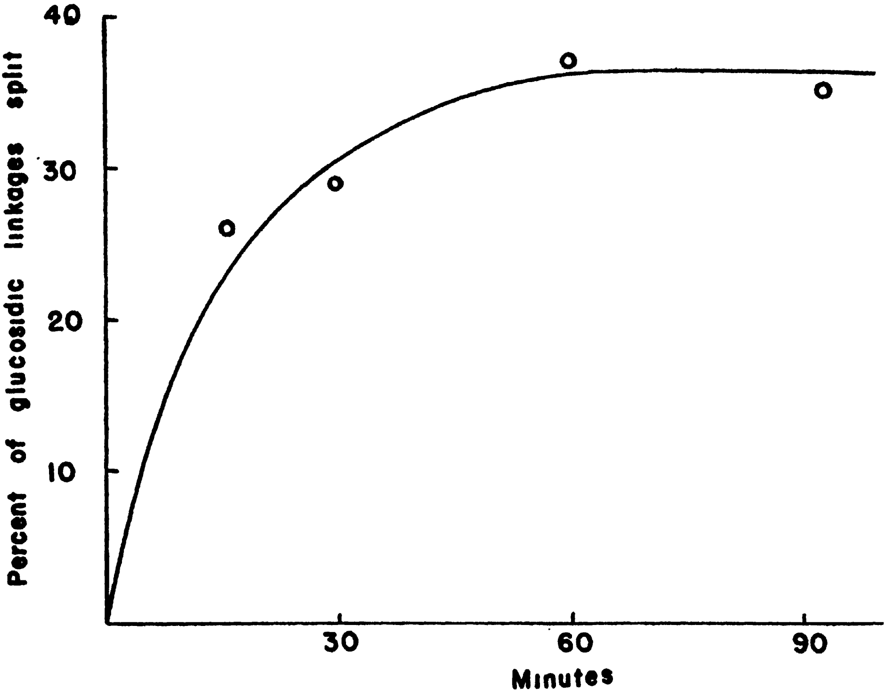

FIG. 2. Phosphorolysis of glycogen in presence of phosphorylase and phosphoglucomutase. The reaction mixture contained 0.02 M potassium phosphate, 0.015 M glycogen Specimen 1 (expressed as glucose), 1 x 10^{-4} M magnesium chloride, 7 × 10^{-3} M cysteine, and 1.5 × 10^{-4} M muscle adenylic acid, pH 7.1. The concentration of crystalline phosphoglucomutase and phosphorylase was 25 and 50 γ per ml., respectively. The per cent phosphorolysis was calculated from the disappearance of inorganic phosphate.

The findings with branched starch fractions (amylopectins) of different

TABLE I

Phosphorolysis of Branched Fraction of Starches by Muscle Phosphorylase

Composition of reaction mixtures and methods as in Fig. 1. Polysaccharide concentration as glucose, 0.006 M. Enzyme dilution, 1:50.

Polysaccharide	Per cent phosphorolysis after incubation for				
	5 min.	10 min.	30 min.	60 min.	100 min.
White potato amylopectin	38		43	44	
Corn amylopectin (β fraction of Schoch)		36	38	38	40
Wheat amylopectin		39	39	41	41
Neisseria perflava "amylopectin"	31		39	39	
Sweet potato amylopectin			33		36

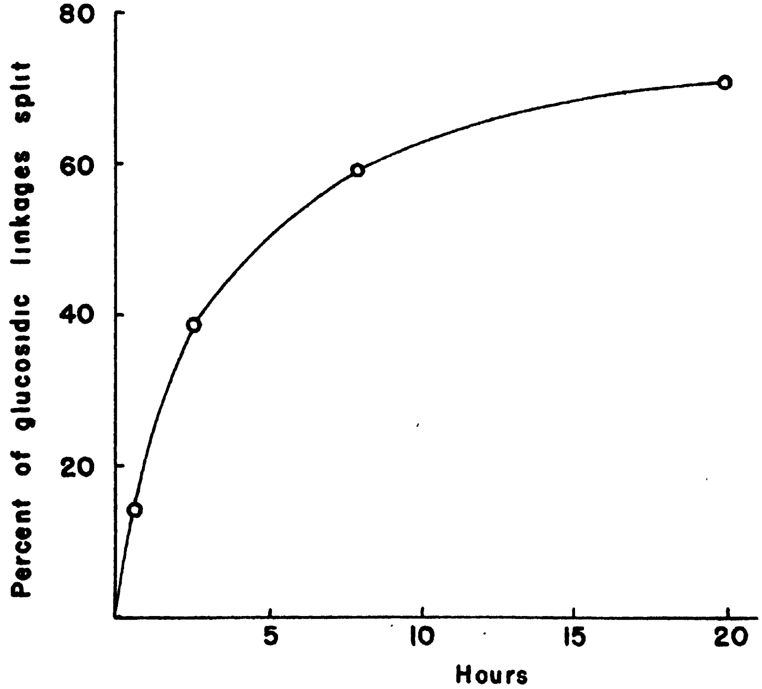

FIG. 3. Phosphorolysis of corn amylose by muscle phosphorylase. Amylose solution in 0.1 M potassium hydroxide was neutralized immediately before use; final concentration as glucose, 0.008 M; enzyme dilution 1:15.

origin are summarized in Table I. An apparent limit of degradation, corresponding to 36 to 44 per cent phosphorolysis, was reached, a result similar to that obtained with glycogen.

Amylose—That the linear fraction of corn-starch can be degraded to a much larger extent than the branched fraction is shown in Fig. 3. The reaction was slow in spite of a high enzyme concentration, presumably because of the low concentration of end-groups in a solution of a linear polymer. The limit of degradation may therefore not have been reached. Another factor to be considered here is the tendency of corn amylose to precipitate (retrogade) from solution. Katz, Hassid, and Doudoroff (3) have, in fact, found almost complete degradation of amylose with potato phosphorylase.

In order to test the phosphorolysis of short chains, amylose was hydrolyzed to the extent of 33 per cent by salivary amylase. After heating the solution to stop the action of amylase, phosphorylase was added. The final yield of 1-ester was equivalent to only 8 per cent of the original amylose. This is consistent with the conclusion that maltose and relatively short amylose chains, those 3 to 4 glucose units long, are resistant to the action of muscle phosphorylase.

Polysaccharides with Terminal or Near Terminal 1,6 Linkages—The following polysaccharides have been examined: a bacterial dextran, LD (glycogen; phosphorylase), LD (glycogen; β-amylase), and several varieties of LD (amylopectin; β-amylase), the amylopectins having been prepared from wheat, corn, white potato, and sweet potato. Tests were carried out under the same conditions as described in the experiments in Fig. 1. With dextran, no 1-ester was formed. With the phosphorylase limit dextrin and the five β-amylase limit dextrins the limit of phosphorolysis was 2 per cent or less.

Degradation by β-Amylase—The degradation of glycogen and amylopectin proceeds further with β-amylase than it does with phosphorylase. This had previously been shown by Swanson (2) for potato phosphorylase. The limit dextrin formed from glycogen by muscle phosphorylase was degraded by β-amylase to maltose to an extent of 24 per cent, while the parent glycogen was degraded to an extent of 47 per cent. On the basis of the structure of glycogen proposed by Meyer (14), it can be assumed that the outer stubs of LD (glycogen; muscle phosphorylase) are 3 glucose units long. This is the minimum chain length required for the formation of 1 molecule of maltose per stub through the action of β-amylase, and it is consistent both with the value of the observed limit of phosphorolysis of glycogen and with the amount of maltose formed from phosphorylase limit dextrin by β-amylase.

Priming Ability of Polysaccharides—As shown in Fig. 4, the limit dextrin prepared from glycogen by exhaustive treatment with β-amylase had a negligible priming effect for muscle phosphorylase. On the other hand, the limit dextrin prepared from glycogen by exhaustive treatment with phosphorylase was about one-half as active as an equal weight of glycogen. The difference can be ascribed to the different lengths of the outer stubs of the two limit dextrins, as discussed in the preceding section. The influence of chain length on priming ability could also be shown in an experiment with glycogen which had been incompletely (31 per cent) degraded by muscle phosphorylase. This material contained only 6 per cent of linkages which could be split by phosphorolysis; in a concentration of 60 mg. per cent it was as effective as primer for muscle phosphorylase as an equal weight of glycogen.

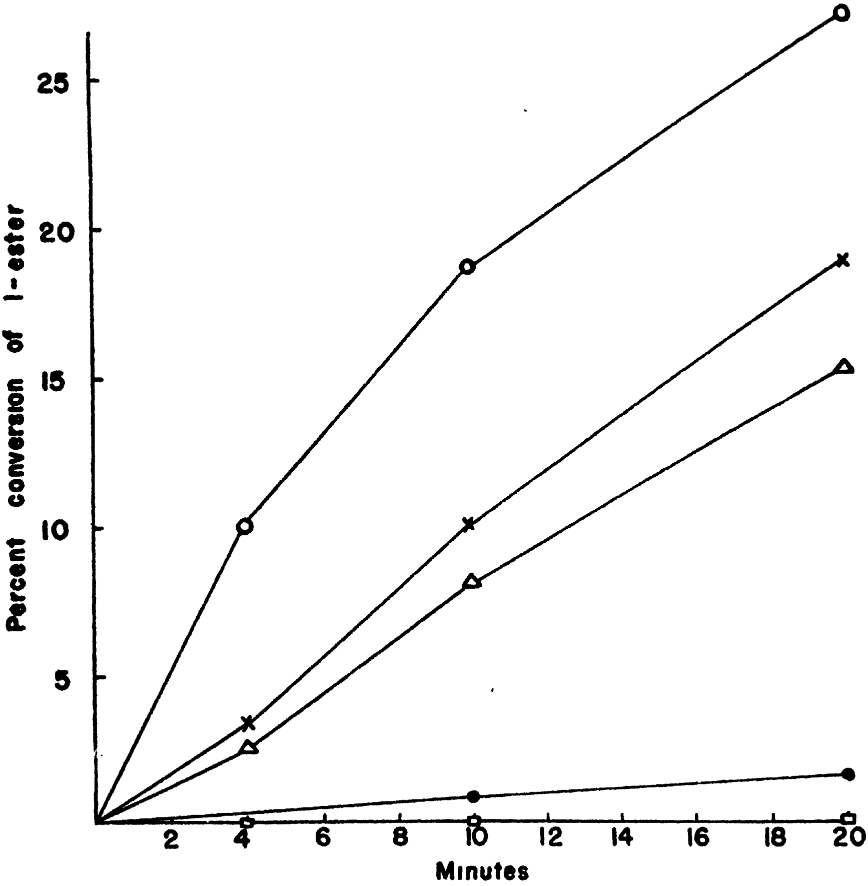

Fig. 4. Glycogen limit dextrins as primers of muscle phosphorylase. Reaction mixtures contained 0.016 M 1-ester and 0.015 M cysteine. Enzyme dilution 1:200, pH 6.8. □ no primer added; ● 240 mg. per cent of LD (glycogen; β-amylase); △ 40 mg. per cent of LD (glycogen; muscle phosphorylase); × 60 mg. per cent of LD (glycogen; muscle phosphorylase); ○ 60 mg. per cent of glycogen.

Potato phosphorylase, in contrast to muscle phosphorylase, can be activated by short linear chains; for example, those formed by partial acid hydrolysis of the cyclic Schardinger dextrins (15). In the case of the α-dextrin the longest chains cannot be more than 6 glucose units. In order to determine whether or not the reducing ends of the chains are essential for the priming effect, they were oxidized with iodine as described under methods. The experiment in Table II shows that the chains with a carboxylic terminal were nearly as effective as primers as those with an aldehydic terminal. Both were transformed into longer chains which gave a blue color with iodine. The addition of glucose units must therefore have occurred at the non-reducing end of the chains.

Since there is disagreement in the literature as to the priming ability of dextrans (16–18), some additional experiments were undertaken with the following materials: a viscous dextran ($[\alpha]_D$ in N NaOH, 200–203°), its acid hydrolysis products with average degrees of polymerization of 55, 35, and 15, respectively, and a dextran with low viscosity obtained from an autolyzed bacterial culture. The materials were tested both directly and after dispersal in 3 N potassium hydroxide, followed by neutralization, in concentrations as high as 500 mg. per cent. In no case was any priming effect observed with these materials on either muscle or potato phosphorylase.

TABLE II

Priming Action of Dextrinic Acids on Potato Phosphorylase

Composition of reaction mixtures: 1-ester, 0.03 M; citrate buffer, pH 6.0, 0.05 M; primer, 30 mg. per cent in terms of original weight of α-Schardinger dextrin; phosphorylase, 0.1 ml. of enzyme solution in 1.0 ml. of reaction mixture. With 100 mg. per cent of soluble starch as primer, 8 μM of inorganic phosphate per ml. were liberated in 10 minutes.

Primer	Color with iodine after incubation for 12 min.*	Inorganic phosphate, μM per ml., after incubation for		
		0 min.	5 min.	12 min.
None	None	0.3	0.3	0.3
Hydrolysate of α-dextrin	Blue	0.3	3.0	6.6
Oxidized hydrolysate of α-dextrin	"	0.3	2.5	5.2

* At zero time, there was no color formation with iodine.

A dextran isolated by Hassid and Barker (19), and previously tested in this laboratory, was again found to activate potato phosphorylase, when used in high concentration after dispersal in alkali.

Effect of LD (Glycogen; Phosphorylase) on Equilibrium—The fact that LD (glycogen; phosphorylase) can act as primer but cannot be degraded by phosphorylase offered an opportunity for testing the theory that polysaccharide synthesis consists in the addition of glucose units to primer end-groups (20). In the formulation shown below, the following abbreviations will be used. G_{n+s} represents a branched polysaccharide with outer chains which consist on an average of $n + x$ glucose residues in α-1,4 linkage, while G_n represents the corresponding limit dextrin whose outer chains are n glucose residues long. As discussed in a preceding section, n probably has the value of 3 in the case of the phosphorylase limit dextrin of glycogen. Glucose-1-phosphate and inorganic phosphate are represented by G-1-P and P, respectively. The conversion of G_n to G_{n+x} can be visualized as a series of successive reversible reactions with equilibrium constants designated as k_1, k_2, k_3, etc.

$$\begin{array}{ll}
G_n + \text{G-1-P} \rightleftharpoons G_{n+1} + \text{P} & k_1 = \dfrac{[G_{n+1}][\text{P}]}{[G_n][\text{G-1-P}]} \\[2ex]
G_{n+1} + \text{G-1-P} \rightleftharpoons G_{n+2} + \text{P} & k_2 = \dfrac{[G_{n+2}][\text{P}]}{[G_{n+1}][\text{G-1-P}]} \\[2ex]
G_{n+2} + \text{G-1-P} \rightleftharpoons G_{n+3} + \text{P} & k_3 = \dfrac{[G_{n+3}][\text{P}]}{[G_{n+2}][\text{G-1-P}]} \\
\cdot & \cdot \\
\cdot & \cdot \\
\cdot & \cdot \\
G_{n+(x-1)} + \text{G-1-P} \rightleftharpoons G_{n+x} + \text{P} & k_x \quad \dfrac{[G_{n+x}][\text{P}]}{[G_{n+(x-1)}][\text{G-1-P}]}
\end{array}$$

It may be seen that k_1 contains the term $[G_{n+1}]$ which cancels a corresponding term in k_2 and so on, so that the equilibrium constant of the over-all reaction will be[4]

$$k_1 \times k_2 \times k_3 \ldots \times k_x = \frac{[G_{n+x}][\text{P}]^x}{[G_n][\text{G-1-P}]^x}$$

Previous experiments (21, 16) have shown that the ratio of inorganic phosphate to glucose-1-phosphate at reaction equilibrium in the presence of muscle phosphorylase is not changed when the concentration of added glycogen is varied between 0.02 and 1 per cent. It has been observed similarly that the ratio, inorganic phosphate to glucose-1-phosphate, at reaction equilibrium in the presence of potato phosphorylase is independent of the concentration of added starch within wide limits (9). This may be explained by the fact that the limits for the general equation ($x = 1$ or $x = 200$) were not approached when equilibrium was reached. Starting with glycogen, $n + x$ is approximately 6, and as shown above, for reactions intermediate between k_1 and k_x there is a cancellation of successive terms, so that the concentration of added polysaccharide does not enter into the calculation of the equilibrium constant. Expressed in other words, one might say that within certain limits the concentration of end-groups remains the same whether the outer chains of glycogen or starch are made longer or shorter by the addition or removal of glucose units.

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[4] The writer is indebted to Dr. M. Cohn for suggestions relating to this equation.

The two terms which do not cancel out and therefore should have an effect on the equilibrium are G_n and G_{n+x} (x being a large number). An analysis of the effect of the term G_{n+x} is made difficult by the fact that, when very long polysaccharide chains are formed, they precipitate out of solution. It seems probable, however, in analogy with the formation of other polymers, that chain length *per se* becomes a limiting factor. In previous experiments the maximal chain length which could be attained with glycogen as primer was about 200 (15).

The special case represented by $x = 1$ with the limit dextrin, G_n, as primer may now be examined. The concentration of G_n (in terms of primer end-groups) has to be equal to or greater than the initial concentration of glucose-1-phosphate, so that at most one chain unit can be added for each available primer end-group. One would then expect a shift in the equilibrium toward the side of polysaccharide synthesis. Furthermore, there should be in this special case a dependence of the equilibrium on the concentration of added primer. Experiments designed to test these predictions are shown in Table III.

In Experiment 1, the initial ratio, inorganic P:1-ester P, was set at 4.8. No reaction took place during incubation with phosphorylase without added primer. From previous determinations of the equilibrium ratio at the particular pH used, one could expect a shift towards a lower ratio; that is, some inorganic phosphate would be removed in the conversion of glycogen to glucose-1-phosphate. This expectation was fulfilled when glycogen was used as primer. In contrast, with LD (glycogen; phosphorylase) as primer the reaction proceeded in the opposite direction; that is, polysaccharide was being synthesized instead of being broken down. Consequently, glucose-1-phosphate disappeared and inorganic phosphate was formed, so that the terminal ratio increased very markedly over that observed with glycogen as primer.

In Experiment 2 the initial ratio, inorganic P:1-ester P, was such that with glycogen as primer a shift to a higher ratio was to be expected. Priming with either polysaccharide brought about polysaccharide synthesis, but with the limit dextrin as primer the terminal ratio was again much higher than with glycogen as primer.

Experiments 1 and 2 show also that the terminal ratio, inorganic P:1-ester P is influenced by the concentration of added limit dextrin. Assuming that LD (glycogen; phosphorylase) contains 14 per cent terminal glucose units, the molar concentration of these can be calculated. This permits one to approximate the equilibrium constant, k_1, as follows. At the start of the reaction there are no chain units present in the limit dextrin which can be split off by phosphorolysis, while at equilibrium the number of chain units which can be removed by phosphorolysis corresponds to the number of glucose-1-phosphate molecules which have disappeared. Hence,

$$k_1 = \frac{[\Delta \text{ G-1-P}][\text{P}]}{[\text{G}_n][\text{G-1-P}]}$$

k_1 calculated in this manner from the data of Table III yielded 1.25, 1.35, and 1.56 for concentrations of limit dextrin of 1.0, 0.5, and 0.25 per cent respectively.

Table III

Effect of Polysaccharides on Ratio of Inorganic Phosphate to Glucose-1-phosphate at Equilibrium

Reaction mixtures consisted of potassium phosphate, dipotassium salt of 1-ester, 0.01 M cysteine, polysaccharide, and muscle phosphorylase diluted 1:50. pH 7.3. 1 ml. samples were treated with 3 ml. of magnesia mixture to remove inorganic phosphate, the suspensions being filtered after standing for 10 hours. Glucose-1-phosphate was determined in aliquots of the filtrates as phosphorus hydrolyzed in 7 minutes in N H_2SO_4 at 100°. The phosphate values are given in micromoles per ml.

Experiment No.	Polysaccharide added, per cent glucose	Inorganic phosphate after incubation for			Glucose-1-phosphate after incubation for		Inorganic phosphate / Glucose-1-phosphate	
		0 min.	30 min.	60 min.	0 min.	30 min.	Initial	Terminal
1	None	11.2	11.2	11.2	2.34	2.40	4.8	4.7
	Glycogen, 1.00	11.1	10.7	10.8	2.25	2.49	4.9	4.3
	LD (glycogen; phosphorylase), 1.00	12.0	13.3	13.0	2.38	1.36	5.0	9.6
2	None	2.60	2.60	2.60	1.05	1.03	2.5	2.5
	Glycogen, 0.50	2.60	2.98	2.96	1.05	0.75	2.5	3.9
	LD (glycogen; phosphorylase), 0.50	3.00	3.66	3.72	1.06	0.44	2.8	8.4
	LD (glycogen; phosphorylase), 0.25	2.71	3.20	3.22	1.03	0.53	2.6	6.1

These results give further support to the theory (20) that polysaccharides act as primers in the direction of synthesis because they enter stoichiometrically into the reaction catalyzed by phosphorylase.

It is a privilege to acknowledge the guidance of Professor C. F. Cori and Professor G. T. Cori in the conduct of this investigation.

SUMMARY

1. Branched polysaccharides (glycogen, amylopectin fractions of several seed and tuber starches, and a bacterial amylopectin) could only be degraded partially (40 ± 5 per cent) by recrystallized muscle phosphorylase, while the degradation of the linear fraction of corn-starch neared

completion after long incubation. Bacterial dextrans and limit dextrins formed from branched polysaccharides by β-amylase were not degraded significantly by recrystallized muscle phosphorylase.

2. The limit dextrin of glycogen, formed by exhaustive action of muscle phosphorylase, could still be hydrolyzed by β-amylase to an extent of 24 per cent, or roughly an equivalent of 1 mole of maltose per calculated end-group. The priming effect of glycogen disappeared after treatment with β-amylase but not after treatment with muscle phosphorylase. These results suggest that the outer stubs of the phosphorylase limit dextrin of glycogen are at least 3 glucose units long, and that this is the minimum chain length required for priming effect. Native and partially degraded specimens of a bacterial dextran failed to prime muscle phosphorylase. Oxidation of the terminal aldehydic group had little effect on the priming ability of short amylose chains for potato phosphorylase.

3. The phosphorylase limit dextrin of glycogen, G_n, was used as primer in a concentration (in terms of end-groups) equal to or greater than the initial concentration of glucose-1-phosphate, G-1-P. In this special case, the ratio, P:G-1-P, at equilibrium of the reaction, G_n + G-1-P $\rightleftharpoons G_{n+1}$ + P, was shown to depend on the concentration of added primer. The value of the equilibrium constant for the above reaction varied between 1.3 and 1.5 when the concentration of added primer was varied 4-fold. This indicates that the primer end-groups enter stoichiometrically into the reaction catalyzed by phosphorylase.

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OXIDATION OF FATTY ACIDS AND TRICARBOXYLIC ACID CYCLE INTERMEDIATES BY ISOLATED RAT LIVER MITOCHONDRIA*

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Beginning with the fundamental observation of Warburg in 1913 (1) it has been a general finding that the more highly organized enzyme systems of animal tissues responsible for oxidation of metabolites by molecular oxygen are associated with the insoluble particulate portion of the cell. Among the several approaches which have been used to study the morphology and composition of such catalytically active particulate material, the most fruitful has been the differential centrifugation technique for separation of nuclei, mitochondria or "large granules," and other substructures developed by Bensley and his school (2) and refined by Claude (3, 4) and Hogeboom, Schneider, and Pallade (5, 6). Considerable work on the composition and enzymatic activity of the various particulate fractions has been described by the Rockefeller group (7, 8), Schneider (9), and other investigators. For instance, quantitative assays have revealed that most of the succinoxidase and cytochrome oxidase activity is present in the mitochondria or "large granules" (7).

In this laboratory studies have been made on the enzymatic oxidation of fatty acids to acetoacetate and also via the Krebs tricarboxylic acid cycle. These complex and highly organized reactions take place in suspensions of particulate material separated from rat liver homogenates by centrifugation (10, 11). Certain observations on the properties of this enzyme system (11) suggested that the activity was to some extent dependent on osmotic factors, and Potter, on the basis of measurements of "cytolysis quotients," suggested that the activity was present only in intact cells (12).

With the publication of what appears to be a definitive method for the isolation of mitochondria or "large granules" by Hogeboom, Schneider, and Pallade (5, 6), it was possible to demonstrate that mitochondria isolated by this method bear all the demonstrable fatty acid oxidase activity of whole rat liver. The particulate material isolated by this method is stated to be homogeneous and identical in morphology and vital staining characteristics

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with the mitochondria of the intact cell (6); mitochondria isolated by the earlier procedures of Bensley and Hoerr (2) and Claude (3) apparently represent partially damaged forms without these properties.

Since the publication of our preliminary note on the localization of fatty acid oxidase activity in these particles (13), Schneider has published a confirmatory report (14). This paper is concerned with the experimental details of the basic experiments.

EXPERIMENTAL

Analytical Methods—Octanoate was determined by the method of Lehninger and Smith (15), acetoacetate by a modification of the method of Greenberg and Lester (16), and citrate by the method of Speck, Moulder, and Evans (17). Manometric measurements of oxygen uptake were made at 30° in Warburg vessels of conventional design with air as the gas phase. Flasks were equilibrated for 5 minutes prior to closing of the taps. Determinations of inorganic and total phosphorus were made according to the method of Gomori (18) and partition of the phosphorus of the enzyme preparations was carried out according to methods described by Schneider (19) and Schmidt and Thannhauser (20). Radioactivity measurements were made on thin layers of aqueous solutions by means of a Geiger-Müller counting tube and recording apparatus of standard commercial type. Separations of esterified phosphate for these measurements were performed as described elsewhere (21).

Preparation of Mitochondria from Rat Liver—The procedure of Hogeboom, Schneider, and Pallade (5, 6) was used for the preparation of the particulate fractions of rat liver. Normal adult albino rats of Sprague-Dawley stock were used throughout this study. The animals were killed by decapitation and exsanguinated. The livers were quickly removed and chilled in cracked ice. All operations during the preparation of the fractions were carried out in a room maintained at 2° and all reagents and apparatus were previously chilled. The fresh, chilled rat liver was homogenized in 9 volumes of cold 0.88 M sucrose in a glass homogenizer of the type described by Potter and Elvehjem (22). Nuclei, whole cells, stroma, and erythrocytes were removed by three successive centrifugations, each of 3 minutes duration, at about 1500 × g in the Sorvall model SP centrifuge. The mitochondria were then sedimented from the cleared supernatant by centrifugation in a Sorvall model SS-1 centrifuge at 18,000 × g for 20 minutes. The sedimented mitochondria were washed by resuspension in 10 volumes of 0.88 M sucrose, followed by resedimentation for 20 minutes at 18,000 × g. The supernatant was carefully decanted and the washed mitochondria were taken up in sufficient ice-cold 0.15 M KCl or water (about 5.0 ml. for each gm. of whole tissue used as starting material) to yield a suspension containing about 1 mg. of total nitrogen per ml. The final concentration of KCl in the fatty acid oxidase test system was about 0.05 M, a value shown to be near the optimum for fatty acid oxidation in a previous study (11). In experiments in which the mitochondria were taken up in distilled water, sufficient KCl was added to the test flasks to provide a final concentration of about 0.05 M.

Throughout these fractionations, it was found essential that low temperatures be maintained in order to preserve enzyme activity. We have found that the Sorvall angle centrifuges are especially well adapted for this purpose, since the temperature rise during centrifugation in the cold room is held to a minimum. The International refrigerated centrifuge has also been used with complete success. Although these fractions can be obtained at higher temperatures, their ability to oxidize fatty acids and Krebs cycle intermediates then becomes greatly attenuated or lost, probably because of enzymatic destruction of as yet unidentified cofactors.

Microscopic examination showed that the mitochondria so prepared were free of whole cells, nuclei, and débris, confirming the work of Hogeboom *et al.* who have stated that this procedure yields morphologically intact mitochondria free of extraneous elements (6). We have found that these preparations are contaminated to a small degree with erythrocytes. These extraneous elements may be removed by taking up the unwashed pellet of mitochondria which had been sedimented once in 10 volumes of 0.88 M sucrose as described above, and subjecting the suspension at this point to two or three preliminary sedimentations at low speed (2000 × *g*), each of 5 minutes duration. The main bulk of the mitochondria, now freed of red blood cells, is then sedimented by means of a 20 minute centrifugation at 18,000 × *g*. This procedure also reduces the desoxypentose nucleic acid phosphorus content of the mitochondria preparations to vanishingly small values. The phosphorus distribution in the mitochondria is discussed more fully in a later section of this paper.

To avoid the necessity of a high speed centrifuge for the preparation of mitochondria, we have also used an abridged procedure which yields preparations of mitochondria which are entirely satisfactory for the study of the enzyme systems involved in this report. The 0.88 M sucrose extract of rat liver, freed of nuclei and whole cells exactly as described above, is sedimented at 2400 × *g* for 30 minutes at 0° in the Sorvall model SP angle centrifuge. The supernatant is decanted and the mitochondria are then washed by resuspension in 10 volumes of 0.15 M KCl and resedimented by centrifugation for 7 minutes at 2400 × *g*. While the yield of mitochondria obtained by this procedure is not so large as in the standard procedure, the material appears to be identical in composition and enzymatic activity. A second abridged procedure has also been used for preparing mitochondria.

The 0.88 M sucrose extract of rat liver, after removal of nuclei, etc., by preliminary centrifugations as outlined by Hogeboom *et al.*, is treated with 0.1 volume of 1.5 M KCl and allowed to stand in an ice bath for 5 to 10 minutes. The addition of salt causes agglutination of a large part of the mitochondria and they are now sedimentable in 5 to 10 minutes at 2000 × *g*. The sedimented material can then be washed with 0.15 M KCl solution to free it of sucrose. Such material appears to be identical in enzymatic behavior with the material obtained by the original method of Hogeboom *et al.* and is obviously more convenient to prepare.

All experiments reported in this paper were done with mitochondria prepared by the original method of Hogeboom *et al.* with or without the additional low speed centrifugations to remove extraneous erythrocytes.

Distribution of Fatty Acid Oxidase Activity in Particulate Fractions of Rat Liver—The three principal fractions obtained from 0.88 M sucrose homogenates by the procedure of Hogeboom *et al.* described above were tested for fatty acid oxidase activity. Sodium octanoate was used as substrate in the standard test system described previously (11). The fractions tested were the "nuclear precipitate," containing principally nuclei, whole cells, erythrocytes, and stroma, together with some mitochondria; the mitochondria or "large granules" of the rat liver, in a purified condition almost entirely free of extraneous structures; and the supernatant, containing "microsomes" (6), ultramicroscopic particles, and the soluble material of the rat liver homogenates. In these experiments, the "nuclear precipitate" was washed once with ice-cold isotonic KCl to free it of oxidizable metabolites. No attempt was made to free the final supernatant of sucrose, which was thus present in the flasks at about isotonic concentrations. Previous work has shown that this concentration of sucrose is somewhat inhibitory to the oxidation.

Typical results of such tests are summarized in Table I. It is seen that only the mitochondrial fraction has the ability to oxidize octanoate as evidenced by octanoate disappearance, oxygen uptake, and acetoacetate formation. In comparable amounts as judged by total nitrogen determination, the "nuclear fraction" and the supernatant were quite inactive in fatty acid oxidation. Furthermore, other experiments in which suspensions of mitochondria were tested in combination with the nuclear fraction and with the supernatant indicated that there was no stimulation of fatty acid oxidation over that shown by the mitochondria alone, nor was there any significant inhibition. Schneider (14) has found that the addition of either the nuclear fraction or the supernatant or both to the mitochondrial fraction increased the activity of the latter slightly. However, Schneider did not add to his test system catalytic amounts of the C_4-dicarboxylic acids needed for the full activity of the fatty acid oxidase system (11).

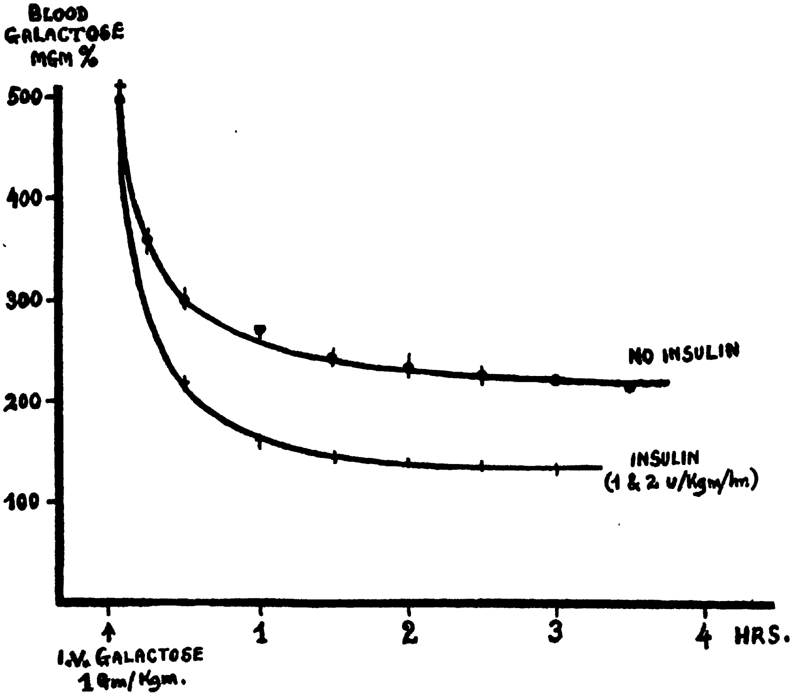

FIG. 1. Blood galactose levels following intravenous administration of galactose to eviscerated, nephrectomized dogs. Insulin was started ½ hour before galactose administration and continued throughout the experiment. All the dogs received intravenous glucose (0.125 to 0.25 gm. per kilo per hour in 26 cc. of saline).

If this hypothesis is confirmed, it opens the question of mechanism by which a molecule of the insulin type may affect the transfer of substances.

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THE ACTION OF INSULIN ON THE DISTRIBUTION OF GALACTOSE IN EVISCERATED NEPHRECTOMIZED DOGS*

Sirs:

The intimate mechanism by which insulin acts has been looked for in a "biocatalytic" effect on one or several of the enzymatic steps of the intermediary metabolism of glucose.[1] However, insulin may not exert any direct action whatever upon the known enzyme systems of the accepted carbohydrate scheme, but it may act by promoting the rate of transfer of glucose (and perhaps other substances) across certain cell membranes. Accordingly we are investigating the effect of insulin upon the rate of entry into tissues of substances other than glucose, preferably non-utilizable, organic, naturally occurring compounds.

This note reports data on the effect of insulin upon the rate at which injected galactose is distributed in eviscerated, nephrectomized dogs. Such preparations do not utilize galactose,[2] as is evident from the fact that, after a period of distribution, the level of galactose in the blood remains practically stationary. When insulin (1 unit per kilo) is administered with the galactose, the final stationary level of blood galactose is markedly lower than in its absence (Fig. 1). Without insulin, galactose is distributed in 45 to 47 per cent of the body weight. Insulin increases the volume of distribution of the hexose to about 75 per cent of body weight, a figure close to that of total body water. Doubling the dose of insulin (2 units per kilo) does not lead to any further changes. Total body water is, of course, the upper possible limit to distribution.

The working hypothesis prompted by these data can be stated as follows: Insulin acts upon the cell membranes of certain tissues (skeletal muscle, etc.) in such a manner that the transfer of hexoses (and perhaps other substances) from the extracellular fluid into the cell is facilitated. The intracellular fate of the hexoses depends upon the availability of metabolic systems for their transformation. In the case of galactose no further changes occur. In the case of glucose, dissimilation, glycogen storage, and transformation to fat are secondarily stimulated by the rapidity of its entry into the cell.

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[2] There is also no demonstrable utilization of galactose by rat diaphragm *in vitro*.

the fact that the patient exhibits markedly increased erythropoiesis (reticulocytosis 8 per cent, bone marrow normoblastic). The considerable increase of N^{15} observed in the coproporphyrin I, uroporphyrin I, and stercobilin suggests that they derive from a common pyrrole precursor, which is most likely also the precursor of the hemoglobin protoporphyrin. The very high N^{15} concentration of the coproporphyrin I in the first period and its immediate rapid decrease accompanied by an increase of the N^{15} concentration of the uroporphyrin I suggest the possibility that this is derived from coproporphyrin I as an expression of the metabolic error of porphyria. On the other hand the slower decrease of the N^{15} concentration of the stercobilin and the decrease of the N^{15} concentration of the hemoglobin protoporphyrin after the 11th day indicate that a minority of the stercobilin comes from the hemoglobin protoporphyrin as a result of destruction of the tagged hemoglobin.

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AN ISOTOPIC STUDY OF PORPHYRIN AND HEMOGLOBIN METABOLISM IN A CASE OF PORPHYRIA*

Sirs:

It has been shown that glycine is a specific precursor of hemoglobin protoporphyrin of rats,[1] dogs,[2] and humans,[3] of the coproporphyrin I of dogs,[2] and of stercobilin in humans.[4]

Particular interest has attached to a case of "light-sensitive" (congenital) porphyria, in which hemoglobin and porphyrin metabolism has been studied with the aid of glycine containing N^{15}.

Time	Hb protoporphyrin N^{15} concentration	Coproporphyrin I N^{15} concentration	Uroporphyrin I N^{15} concentration	Stercobilin N^{15} concentration
days	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*
4		5.686 5.614*	2.918	1.057
7	1.006			
8		2.594	3.234	2.991
11	1.146			
12		0.897	1.321	2.234
15	1.057			
16		0.484	0.765	0.926
18	0.941			

* N^{15} concentration of coproporphyrin I isolated from feces.

A girl of 4 years, exhibiting photosensitivity, anemia, and splenomegaly, excretes large amounts of coproporphyrin I, uroporphyrin I, and stercobilinogen. She was given 15 gm. of glycine containing 32.2 atom per cent excess N^{15} in divided doses over a 3 day period. Serial determinations[5] were then made of the N^{15} in the hemoglobin protoporphyrin, coproporphyrin I and uroporphyrin I of the urine, and the stercobilin of the feces. The most significant of the results thus far obtained are shown in the accompanying table. It can be seen that the uptake of N^{15} by the hemoglobin protoporphyrin is considerably greater than that previously recorded for a normal subject.[3] This is probably due to base. The weight of purine or pyrimidine in each fraction is calculated from the absorption of known solutions of the compounds.

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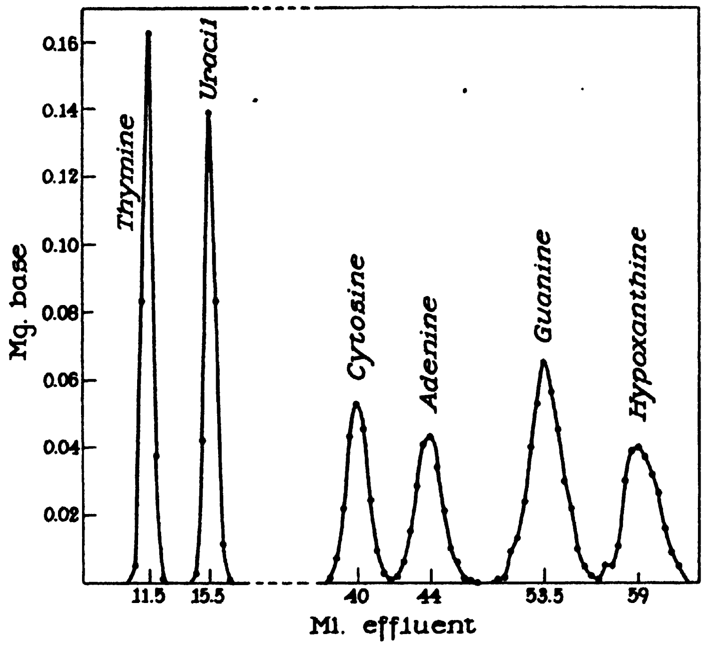
[5] These were carried out by means of the mass spectrometer in Dr. Alfred O. C. Nier's laboratory.

If the extinction coefficient or the weight of base in each fraction is plotted against effluent volume, a series of sharp, well separated peaks is obtained (see the figure). The curves are integrated by the addition of the analytical values for the points on a given peak. For accurate plotting and integration, an average fraction ahead of or behind the peaks should be used as the blank for measurement of the extinction coefficients. The positions of the peaks are reproducible to ±10 per cent.



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LETTERS TO THE EDITORS

CHROMATOGRAPHY OF PURINES AND PYRIMIDINES ON STARCH COLUMNS

Sirs:

We have found that it is possible to obtain satisfactory resolution of the six bases, thymine, uracil, cytosine, adenine, guanine, and hypoxanthine, by chromatography on a single column of the type developed by Stein and Moore[1] for amino acids. On known mixtures recoveries have averaged 100 ± 4 per cent. The bases are easily identified by their absorption spectra which are characteristic, and not appreciably changed by passage through the column. The quantitative estimation of purines and pyrimidines by paper chromatography has been described by Vischer and Chargaff[2] and by Hotchkiss;[3] and Edman *et al.*[4] have reported the separation of adenine and guanine on a starch column.

The starch columns employed in our work are 30 cm. in height and 0.9 cm. in diameter, and are prepared according to the procedure recommended by Stein and Moore. The solvent is composed of *n*-propanol and 0.5 N HCl in the proportions of 2:1. A solution containing from 0.20 to 0.35 mg. of each base in 0.5 to 1.0 ml. of solvent is added to the top of the column. The effluent is collected in a regular series of 0.5 ml. fractions.[5]

The fractions are evaporated to dryness in groups of 50 to 80 in a vacuum desiccator warmed to about 40° by means of an infra-red lamp. Each residue is dissolved in 5 ml. of 0.1 N HCl. The extinction coefficients of the solutions are measured in the Beckman spectrophotometer at wave-lengths corresponding to the absorption maximum for each

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[5] The procedure for the addition of the sample to the column and the collection of the effluent is the same as that described by Stein and Moore. The authors wish to acknowledge many helpful suggestions from Dr. Moore and Dr. Stein which have aided in carrying out this work.

have demonstrated the presence of such a component in about 60 per cent of rabbit sera.

SUMMARY

1. The difficulties which have prevented a satisfactory interpretation of the ultracentrifugal pattern of human serum, both diluted and undiluted, have been reviewed.

2. The observation of a "dip" in the ultracentrifugal pattern of undiluted human sera has led the present authors to explain the major peculiarities of albumin boundary asymmetry as being due to a pile up of lipoprotein (X protein) on the albumin concentration gradient. The existence of the pile up phenomenon renders classical two-component resolution of asymmetrical albumin boundaries completely erroneous both in the calculation of sedimentation rates and concentration of lipoprotein.

3. A method for measuring the concentration of low density lipoproteins by flotation has been described and applied. The results of analysis of lipoprotein concentrations by this method are in much better agreement with electrophoretic and fractionation data concerning this lipoprotein than are the data in the literature up to the present. The data obtained in the present work render unnecessary the postulation of a great degree of lability of lipoprotein with variation in salt and protein concentration.

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salt concentrations. The pile up theory presented here could readily explain the apparent changes in concentration of lipoprotein observed by Pedersen without invoking any dissociation of the molecule. In Table II is given the concentration of lipoprotein at various total protein and salt concentrations for a single serum. Within the experimental error of measuring small areas, the data indicate no significant variation of lipoprotein content and hence the stability of this molecule, in accord with the report of Edsall (6) on the relative stability of the low density B_1 lipoprotein to such manipulations as precipitation and resolution.

TABLE I

Low Density Lipoprotein Content of Ten Normal Human Sera

Serum No.	Sp. gr. of serum	Total protein	Lipoprotein	Lipoprotein
		gm. per 100 ml.	*gm. per 100 ml.*	*per cent of total*
1	1.0305	8.10	0.20	2.5
2	1.0310	8.26	0.23	2.8
3	1.0276	7.08	0.40	5.6
4	1.0298	7.86	0.35	4.4
5	1.0314	8.40	0.24	2.9
6	1.0288	7.50	0.23	3.0
7	1.0294	7.70	0.45	5.8
8	1.0278	7.14	0.43	6.0
9	1.0244	5.96	0.33	5.7
10	1.0240	5.82	0.20	3.4

TABLE II

Low Density Lipoprotein Content of a Single Human Serum Sample Obtained from Different Salt and Total Protein Concentrations

Preparation of serum sample	Cell used	Resultant sp. gr.	Lipoprotein	Lipoprotein
	ml.		*gm. per 100 ml.*	*per cent of total protein*
3.0% NaCl added	0.3	1.048	0.33	4.6
4.7% " "	0.3	1.060	0.33	4.7
6.1% " "	0.3	1.069	0.42	6.0
7.8% " "	0.3	1.081	0.40	5.6
9.4% " "	0.3	1.093	0.41	5.8
1 volume serum + 1 volume 8% NaCl	0.8	1.042	0.40	5.6
1 " " + 3 volumes 8% NaCl	0.8	1.048	0.46	6.5

It has been further stated that a density-sensitive component is present only in the sera of humans (7). Ultracentrifugal studies reported elsewhere[1] plied to the albumin boundary is not applicable. Determinations such as those of Pedersen and McFarlane measure, therefore, some function of the concentration of lipoprotein that has piled up in the albumin boundary, but by no means the true serum lipoprotein content.

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[1] Lindgren, F. T., Elliott, H. A., and Gofman, J. W., in preparation.

In view of the low density of the lipoprotein (specific volume 0.97), a method capable of measuring its concentration in human serum is available; namely, flotation. The necessary requirement that the density of the serum be greater than that of the lipoprotein may readily be achieved by the addition of small quantities of sodium chloride. Once the lipoprotein has moved a small distance away from the base of the cell in flotation, it then moves in an essentially homogeneous medium. Fortunately, the rate of flotation can be made sufficiently rapid so that the lipoprotein concentration measurements can be made before its boundary is obscured by meeting the sedimenting protein components. Fig. 4, *a*, *b*, and *c* shows the progressive flotation of the low density lipoprotein, as studied in serum containing 7.8 per cent of added sodium chloride. The lipoprotein appears as an "inverse" peak, which is fully expected from the theory of the optical system. Since the specific refractive increment of the lipoproteins is very close to that for other proteins (5), the area under this peak is a measure of the concentration of the lipoprotein. At the density chosen for these studies, the albumin peak is essentially symmetrical (Fig. 4, *d*). A comparison of the area under the lipoprotein peak with that under the albumin peak gives a measure of the abundance of low density lipoprotein relative to those substances measured ultracentrifugally as albumin.

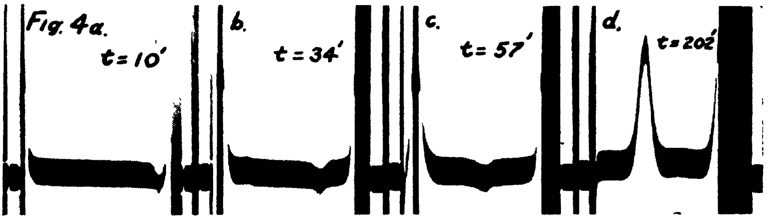

FIG. 4. Progressive flotation of lipoprotein in a serum containing 7.8 per cent added NaCl.

Ten sera from normal male and female young adults were studied by the method of flotation. The results tabulated in Table I indicate the lipoprotein concentration to be of the order of 5 per cent of total serum proteins, an abundance far lower than that quoted in the literature on the basis of previous interpretation of sedimentation diagrams.

The *X* protein has been suggested by Pedersen to be a labile complex of albumin, globulin, and lipides on the basis of changes in the apparent concentration of this component with changes in the serum protein and this instance the lipoprotein sediments with the albumin boundary, it cannot be seen as an independently sedimenting component.

(*b*) The lipoprotein sediments toward the albumin boundary from both sides. This is mandatory if the lipoprotein density falls between the density of albumin solution and that of the supernatant solution. Here, if the lipoprotein sediments more rapidly in supernatant solution than does albumin, an appreciable pile up will be expected. It is this situation which, we believe, usually exists in undiluted serum. Further, small density increments produced by salt or sucrose addition to serum may be expected to shift the pile up along the albumin concentration gradient and thus give rise to a variety of bizarre patterns described in Fig. 3.

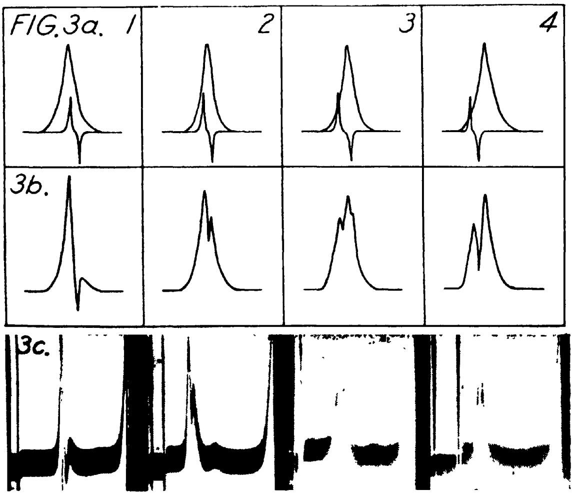

FIG. 3. Variations in ultracentrifugal patterns with variations in the location of the lipoprotein pile up on the albumin concentration gradient.

In the situation described in section *b*, a lipoprotein boundary migrating toward the center of rotation may or may not be observed. If the migration is slow, the boundary may be poor or may be lost entirely, due to the factors usually operative in producing diffuseness of boundaries.

A third situation may arise when the density of the solution is raised to the point where lipoprotein migrates in the direction opposite to albumin both above and below the albumin boundary. In this case, the analysis of Johnston and Ogston applies and predicts the possibility of some distortion of the albumin boundary. However, no pile up phenomenon will be expected along the albumin concentration gradient. It is to be noted here that, for lipoprotein, the viscosity and buoyancy effects operate in opposite directions, so that distortions will tend to be minimized.

It is thus evident that the classical method of boundary resolution apposition of the albumin concentration gradient. Fig. 3, *a* shows the separate albumin and lipoprotein patterns with such relative displacements. Fig. 3, *b* shows the corresponding composite patterns. All these types of distortions of the albumin boundary complex have been observed by altering serum density (see Fig. 3, *c*). Sucrose, sodium chloride, or magnesium sulfate added in quantities sufficient to give equivalent density increments produces the same type of pattern distortion.

The basis for the pile up phenomenon is the difference in sedimentation rates of the lipoprotein on either side of the albumin boundary gradient. A related type of anomaly occurring in mixtures of proteins, without a pile up but due to the same fundamental cause, has been previously described and mathematically treated by Johnston and Ogston (4). Two main factors contribute to this change: The viscosity of the albumin-containing solution is higher than the viscosity of its own supernatant solution, and the effective buoyant force on sedimenting molecules is not the same in the albumin solution as in the supernatant solution, since the density difference between sedimenting particle and sedimenting medium is not the same in the two solutions. This is particularly important for the lipoprotein, the density of which is very close to that of the serum itself.

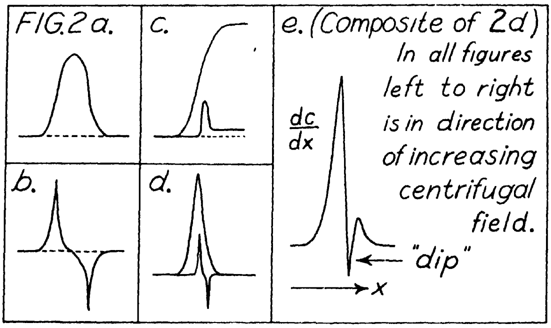
FIG.2a.
c.
e. (Composite of 2d)
In all figures left to right is in direction of increasing centrifugal field.
dc/dx
b.
d.
"dip"
x

FIG. 2. Pile up analysis and the resulting "dip" phenomenon that is observed ultracentrifugally (see the text for a complete explanation).

Two major types of situations can in general result in the pile up of lipoprotein: (*a*) The lipoprotein sediments in the same direction as the albumin. Here buoyancy difference and viscosity difference are additive in slowing lipoprotein sedimentation in the albumin solution relative to that in supernatant solution. Now, if lipoprotein has a sedimentation rate in supernatant solution greater than the albumin sedimentation rate, whereas the lipoprotein in albumin solution sediments more slowly than albumin itself, then the effect will be to produce a progressive pile up of lipoprotein somewhere in the albumin concentration gradient. Since in giving centrifugal fields between 240,000*g* and 300,000*g*, at the meniscus and base, respectively. Blood was obtained from presumably normal individuals in the postabsorptive state.

A large number of undiluted sera were studied ultracentrifugally. Typical patterns obtained approximately 2 hours after reaching full speed are shown in Fig. 1. The vertical bar seen in Fig. 1, *b*, *c*, and *d* in the albumin complex represents a region of refractive index gradient in the cell so great that an entering light beam is completely thrown out of the optical system. We have determined that this bar has no bearing on the symmetry or asymmetry of the albumin peak. Of great interest is the "dip" below the base-line characteristically associated with the asymmetric boundary of the albumin complex. Pedersen accounted for albumin boundary asymmetry as being due to the presence of a density-sensitive lipoprotein (X protein), the $S_{20,w}$ value of which is very close to that of albumin. However, a two-component resolution, assuming a protein of this nature, even if present in the high concentration which Pedersen described, could not conceivably give rise to the "dip" phenomenon which we have observed. An explanation is possible if we assume that the asymmetry and "dip" result from a piling up of the lipoprotein along the albumin concentration gradient at the sedimenting albumin boundary. Fig. 2, *a* gives the concentration diagram for a lipoprotein which for any reason has completely piled up in the region of the albumin gradient. The theory of the diagonal bar-cylindrical lens method of recording refractive index boundaries reveals that such a pile up must give rise to a *biphasic* curve (Fig. 2, *b*). In Fig. 2, *c* is given the concentration diagram for lipoprotein, in process of piling up, and for albumin, and in Fig. 2, *d*, the separate corresponding optical patterns expected. Fig. 2, *e* shows the albumin and lipoprotein pile up patterns in a single composite picture, which is the net result observed with the ultracentrifuge. A comparison of Fig. 2, *e* with the experimental observations of Fig. 1 demonstrates the plausibility of our hypothesis of the origin of the "dip" phenomenon.

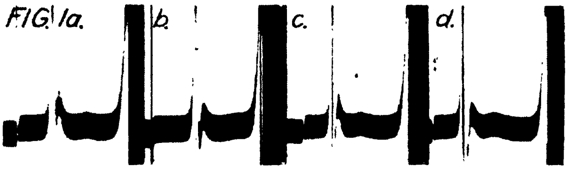

FIG. 1. Ultracentrifugal patterns of normal undiluted human sera obtained approximately 2 hours after the rotor attained full speed.

It is of interest to consider how the observed ultracentrifugal pattern will vary with *slight* displacement of the lipoprotein pile up relative to the

ULTRACENTRIFUGAL STUDIES OF LIPOPROTEINS OF HUMAN SERUM

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In spite of several extensive ultracentrifugal studies of human sera by Mutzenbecher, McFarlane, and Pedersen, the interpretation of the patterns observed has remained in doubt. Specifically, the greatest difficulties encountered by previous workers have arisen in their efforts to study undiluted human sera. McFarlane (1) referred to marked distortions in the pattern observed with undiluted human serum and suggested a trial and error dilution of the serum with salt solution to minimize such distortions. Pedersen (2) recommended diluting sera with various salt or buffer solutions to 40 per cent of the initial concentration, since under these conditions "adequate" resolution of the albumin and so called "*X* protein" peaks in the sedimentation diagram could be made. Both these workers found the apparent concentration of the *X* protein to vary considerably with respect to both over-all protein and salt concentration. The variations in concentration ranged from a value of *X* protein constituting up to 30 per cent of the serum proteins when studied in concentrated serum to an immeasurably small value when the serum was greatly diluted. Pedersen has explained this variation in *X* protein concentration by assuming this molecule to be a labile complex of albumin, globulin, and lipides which dissociates with increasing dilution of the serum.

On the basis of ultracentrifugal studies of human sera by the present authors, a wholly different interpretation of the ultracentrifugal patterns observed is given herewith. This interpretation indicates that the *X* protein concentration in human sera is vastly smaller than reported by Pedersen or McFarlane, but more consistent with electrophoretic data for the low density B_1 lipoproteins. Further, the ultracentrifugal pattern observed for human serum with increasing dilution can be explained without assuming that any dissociation of *X* protein occurs.

EXPERIMENTAL

An electrically driven ultracentrifuge designed by E. G. Pickels was employed, the Thovert-Philpot-Svensson cylindrical lens-refractive index method for the observation of migrating boundaries being utilized (3). All runs were made between 25 and 30° at rotor speeds of 59,780 R.P.M., It is possible that the other fractions contributed trace amounts of such compounds, accounting for the small stimulation observed by Schneider. These preparations of mitochondria showed activity in the oxidation of fatty acid, based on total enzyme nitrogen values, at least as great as the most active preparations obtained previously from isotonic saline homogenates. The Q_{O_2} of such preparations (c.mm. of oxygen taken up per mg. of dry weight per hour at 38° in the presence of octanoate substrate) is approximately 65 to 70. Due to variable losses of activity during preparation, it is probable that these values are not maximal.

TABLE I

Fatty Acid Oxidase Activity of Fractions Prepared from 0.88 M Sucrose Homogenates of Rat Liver

The Warburg vessels contained a final volume of 5.0 ml. The final concentrations of added substances were as follows: KCl, 0.05 M; sodium L-malate, 0.0005 M; $MgSO_4$, 0.005 M; phosphate buffer, pH 7.4, 0.01 M; adenosine triphosphate, 0.0005 M; and cytochrome *c*, 1×10^{-5} M. Water was substituted for sodium octanoate, 0.002 M, in control vessels. The flasks were incubated at 30° for 45 minutes with air as the gas phase. An aqueous suspension of the nuclear precipitates and mitochondrial fractions was added to the respective test systems in amounts representing the yields from 450 mg. of fresh, wet tissue in Experiment 1, and 375 mg. in Experiment 2. The supernatant fraction was derived from 150 mg. of fresh tissue in each experiment.

Experiment No.	Fraction	Enzyme nitrogen	Octanoate	Oxygen uptake	Octanoate disappearance	Acetoacetate formation
		mg.		μM	μM	μM
1	Mitochondria	2.7	−	6.1		0.3
		2.7	+	23.2	8.0	8.5
	"Nuclear ppt."	3.5	−	1.5		0.3
		3.5	+	2.3	0.9	1.1
	Supernatant	4.5	−	1.3		0.2
		4.5	+	1.0		0.2
2	Mitochondria		−	3.0		0.5
			+	16.1	5.1	4.1
	"Nuclear ppt."		−	2.9		0.0
			+	3.4	0.0	0.9
	Supernatant		−	2.9		
			+	3.3		

It was of considerable interest to determine whether the cofactor requirements of the fatty acid oxidase complex in the mitochondria were essentially the same as those previously described (11) for saline-washed particulate material of rat liver. The requirements of the suspensions of mitochondria for added cofactors needed for fatty acid oxidation are summarized in Table II. It can be seen that they are identical with those previously reported, magnesium ions, neutral salt, adenosine triphosphate, cytochrome *c*, and catalytic amounts of malate being necessary for full activity. In the mitochondria, however, the oxidation proceeds at about half the optimum rate in the absence of added cytochrome *c*. The water suspensions of saline-washed enzyme previously studied were almost completely dependent upon added cytochrome *c* for activity (11). This difference is most probably due to the fact that the method used for the preparation of the purified mitochondria allows these structures to be obtained in a more nearly undamaged form, without loss of cytochrome *c* by "leaching out" during the course of the preparation. It should be noted further that a requirement for added KCl or other solute, previously shown to be necessary for the enzyme complex, can be demonstrated for the purified mitochondria only if these particles are first washed free of sucrose, which otherwise completely replaces added KCl, as previous work has shown (11).

TABLE II

Requirements of Fatty Acid Oxidation in Mitochondria

The system is identical with that described in Table I, except that incubation was for 70 minutes. When the components were omitted, water was substituted to maintain the volume constant. The mitochondria were added as an aqueous suspension, containing about 1.3 mg. of enzyme N per flask, after sucrose had been removed by washing with isotonic KCl.

	O_2 uptake	Octanoate disappearance	Acetoacetate formed
	μM	μM	μM
Complete system	12.3	2.7	2.2
Octanoate omitted	2.5		0.5
Malate omitted	0.93	0.2	0.4
$MgSO_4$ "	3.6	0.0	0.6
Cytochrome *c* omitted	8.5	1.3	1.4
KCl omitted	3.2	0.0	0.5
ATP "	0.45	0.0	0.2

Schneider (14) reported that mitochondria isolated from 0.88 M sucrose homogenates showed very low rates of octanoate oxidation. However, he did not supplement his test system with the C_4-dicarboxylic acids. As the data in Table II show, the presence of malate is absolutely essential for oxidation of octanoate in mitochondria prepared by this method and this fact probably explains his failure to find appreciable activity.

Localization of the fatty acid oxidase activity in the mitochondria prepared from 0.88 M sucrose suspensions implies that the saline-washed particulate material previously studied (11) actually was enzymatically active because of its content of mitochondria. In order to settle this point conclusively, a saline-washed preparation of the particulate matter of rat liver was made (11), suspended in 10 volumes of 0.88 M sucrose, and lightly homogenized. This suspension was then subjected to the procedure of Hogeboom *et al.* for obtaining mitochondria from whole liver. Three centrifugations at 2000 × *g* were made to remove nuclei, erythrocytes, and whole cells. The mitochondria remaining in the suspension were then sedimented at high speed and washed as usual. A control preparation of mitochondria made from the same weight of the same rat liver was isolated directly by the technique described in the preceding section. Both suspensions of enzyme were tested for fatty acid oxidase activity. The results are summarized in Table III. The data clearly indicate that the saline-washed particulate enzyme system previously studied contains sufficient particulate material, having sedimentation characteristics identical with those of mitochondria from fresh whole liver extracts, to account for all the fatty acid oxidase activity observed in the crude preparation. The finding that the mitochondria, as isolated by the sucrose procedure from the whole liver cells, are the site of the enzymatic oxidation of fatty acid, and that apparently identical particles can be extracted by the same procedure from particulate rat liver enzyme preparations show identical activity, strongly supports the view that the mitochondria or "large granules" are the sole intracellular structures of the rat liver active in fatty acid oxidation under the test conditions used. It should be stressed that the fatty acid oxidase system is made up of many individual enzymes and is obviously extremely complex. It is conceivable that the nuclear fraction or the supernatant may actually contain some of the individual enzymes neces-

TABLE III

Activity of Mitochondria Isolated from Saline-Washed Enzyme System Compared with Mitochondria Isolated Directly from Fresh Liver

The test system is the same as that in Table I, except that the octanoate concentration was 0.001 M. Water was added to replace octanoate in control flasks, and incubation was for 60 minutes. Mitochondria derived from 375 mg. of fresh tissue were added to each flask.

Mitochondria	Octanoate	Oxygen uptake	Octanoate disappearance	Acetoacetate formation
		μM	*μM*	*μM*
From fresh liver	−	3.3		0.1
	+	15.3	5.0	5.7
Isolated from saline-washed enzyme	−	1.7		0.2
	+	14.9	5.0	4.9

sary for fatty acid oxidation but may be totally deficient in one or more enzymes of the system, causing over-all inactivity.

Oxidation of Krebs Tricarboxylic Acid Cycle Intermediates in Mitochondria—Since the saline-washed particulate material previously studied contains all the enzymes involved in the Krebs cycle and since the mitochondria had previously been shown to contain considerable succinoxidase activity (7), it was of interest to determine whether isolated mitochondria also possessed the enzymatic equipment necessary for the oxidation of pyruvate and other intermediates of the Krebs cycle. The results of a typical experiment in Table IV indicate that intermediate compounds of the Krebs cycle are readily oxidized by suspensions of rat liver mitochondria. The nuclear and microsome fractions showed slight activity, which may have been due to contamination of these fractions by mitochondria. The substrates tested in this experiment were pyruvate plus oxalacetate, citrate, and α-ketoglutarate. These oxidations represent key enzymatic steps of the Krebs cycle. In addition, these mitochondrial preparations are capable

TABLE IV

Activity of Subcellular Fractions of Rat Liver in Oxidation of Intermediate Compounds of Krebs Cycle

The flask contents were as follows: glycylglycine buffer, pH 7.2, 0.033 M; adenosine triphosphate, 0.0005 M; cytochrome *c*, 1 × 10^{-5} M; $MgSO_4$, 0.005 M; 0.1 ml. of orthophosphate containing P^{32}, 359,000 counts per minute (the phosphate esterification data are in Table VI). The final concentration of substrates was 0.01 M in each case, except for oxalacetate and pyruvate which were added together at a concentration of 0.005 M each. Mitochondria and nuclear precipitate fractions were added, so that each flask contained an amount of material derived from 225 mg. of fresh wet liver tissue. The flasks containing supernatant were tested with the material derived from 90 mg. of tissue. The final volume was 3.0 ml.; incubation for 40 minutes with air as gas phase.

Fraction	Substrate	Oxygen uptake
		μM
Mitochondria	Citrate	7.1
	α-Ketoglutarate	6.3
	Pyruvate + oxalacetate	7.1
	None	0.18
"Nuclear ppt."	Citrate	1.9
	α-Ketoglutarate	1.7
	Pyruvate + oxalacetate	0.98
	None	0.0
Supernatant	Citrate	0.54
	α-Ketoglutarate	0.0
	Pyruvate + oxalacetate	1.4
	None	0.31

of catalyzing the condensation of oxalacetate and pyruvate to yield citrate. The experiment summarized in Table V shows aerobic citrate formation from pyruvate when malate served as a source of oxalacetate.

Esterification of Phosphate Coupled to Oxidation in Mitochondrial Preparations—The oxidation of fatty acids and of the intermediate compounds of the Krebs cycle proceeds with the release of considerable amounts of energy. It is now well known that energy released during oxidations over the Krebs cycle may be recovered in part by coupled esterification of inorganic phosphate. More recently it has been shown that oxidation of octanoate by particulate rat liver preparations also caused coupled esterification of inorganic phosphate (11). In order to determine whether the enzymatic equipment necessary for esterification of phosphate coupled to Krebs cycle oxidations and to fatty acid oxidation is present in purified mitochondria, such oxidations were carried out in the presence of inorganic phosphate labeled with P^{32}. At the completion of the incubation, the carrier-diluted inorganic phosphate was removed from the neutralized trichloroacetic acid filtrates by repeated magnesia precipitation (21) and the radioactivity of the esterified phosphorus fractions determined. The data are presented in Table VI. It can be seen from these data that both Krebs cycle oxidations and octanoate oxidation in the suspensions of mitochondria cause extensive incorporation of the P^{32} into the esterified fraction.

TABLE V

Citrate Formation from Malate and Pyruvate in Mitochondria

The flask contents were as follows: 0.05 M KCl, 0.005 M $MgSO_4$, 0.01 M phosphate, pH 7.4, 0.0005 M adenosine triphosphate, and 10^{-5} M cytochrome *c*. The final concentration of malate and pyruvate was 0.01 M in each case, and the final volume was 3.0 ml. Each flask contained mitochondria suspended in 0.15 M KCl equivalent to about 1 mg. of enzyme N. The time of incubation was 65 minutes at 30° with air as the gas phase.

Substrate	Oxygen uptake	Pyruvate used	Citrate formed
	μM	μM	μM
Pyruvate only	10.1	18.4	3.9
Malate only	10.7		2.3
Pyruvate + malate	13.5	9.4	10.4
None	0.5		0.0

Phosphorus Distribution in Purified Mitochondria—Previous workers in describing the chemical constitution of the mitochondria (4, 23) have emphasized the high content of phospholipide in these structures and the fact that they contain nucleic acid of the pentose nucleic acid type. The distribution of phosphorus in a typical preparation of mitochondria made by the standard method of Hogeboom *et al.* (5) is presented in Column 1 of Table VII. Characteristically high values of phospholipide phosphorus, and the predominance of pentose nucleic acid, with only small amounts of desoxypentose nucleic acid, are to be noted. These figures are in fair

TABLE VI

Esterification of Phosphate Coupled to Oxidations in Mitochondria

The conditions of Experiment 1 (Krebs cycle oxidations) were exactly as described for experiments summarized in Table IV. In Experiment 2 (fatty acid oxidation) vessels contained 0.005 M $MgCl_2$, 0.01 M glycylglycine buffer, pH 7.4, 1 × 10^{-5} M cytochrome *c*, 0.001 M adenosine triphosphate, 0.0001 M malate, 0.05 M KCl, 0.001 M octanoate, and inorganic orthophosphate labeled with 157,000 counts per minute of P^{32}. Octanoate was omitted in the control vessel. The time of incubation was 20 minutes at 30°.

Experiment No.	Substrate	O_2 uptake	Esterified P	P^{32} esterified
		μM	γ	*per cent*
1	None	0.18	24.2	0.67
	Citrate	7.1	106	31.3
	α-Ketoglutarate	6.3	113	39.3
	Pyruvate + oxalacetate	7.1	113	32.6
2	None (0.0001 M malate present)	0.5	37	3.2
	Octanoate	4.5	121	27.8

TABLE VII

Distribution of Phosphorus in Mitochondria Derived from 0.88 M Sucrose Homogenates of Rat Livers

Total nucleic acid phosphorus was determined by the method of Schneider (19). Pentose nucleic acid was differentiated from desoxypentose nucleic acid by the method of Schmidt and Thannhauser (20). In Column 1 are listed values obtained for mitochondria isolated by the original method of Hogeboom *et al.* (6). In Column 2, values are given for such mitochondria which had been freed of erythrocytes and other extraneous elements by the modification described in the test.

Fraction	Per cent of total P	
	Method of Hogeboom *et al.* (1)	Modified method (2)
Acid-soluble P	21.3	15
Lipide P	56	56
Total nucleic acid P	19.3	21.3
"Protein" P	6.0	7.2
Desoxypentose nucleic acid P	1.5	0.5
Pentose nucleic acid P	17.8	20.8
PNA-P:DNA-P	11.8	41.6

agreement with those published by Hogeboom *et al.* (6) and Schneider (9). Their data did not include direct measurement of both nucleic acids on the mitochondrial fraction but did show that almost all of the desoxypentose nucleic acid was in the first nuclear precipitate. Our direct analysis of the mitochondria shows the presence of appreciable amounts of DNA,[1] which may be due to contamination by other morphological elements. In Column 2 is given the phosphorus distribution in another preparation of mitochondria which had been subjected to more extensive removal of extraneous elements by repeated centrifugation at low speed prior to the resedimentation of the washed particles at high speed as already described. It can be seen that this procedure has reduced the amount of DNA and raised the ratio of PNA:DNA from 11.8 to 41.6. This ratio represents the analytical limit of the methods of Schneider and Schmidt and Thannhauser for measuring pentose nucleic acid and DNA in our hands. Preparations with very low DNA values thus obtained were found to be active in the oxidation of fatty acids. It is difficult to determine whether the last trace of DNA phosphorus in the preparations studied is analytically significant. It is of course conceivable that trace amounts of DNA are present normally in the mitochondria.

Fatty Acid Oxidase Activity of Mitochondria Obtained by Other Procedures—It was found in the course of this work that the choice of solvents in which the rat liver was homogenized prior to fractionation was of critical importance in the distribution of the fatty acid oxidase activity. When distilled water or distilled water made slightly alkaline by the addition of NaOH ("neutral water") was used as the medium for the preparation of mitochondria and other particulate fractions according to the procedure of Schneider (9), no activity in the oxidation of fatty acid could be detected in any fraction.

Hogeboom *et al.* (6) have stated that "large granules" obtained by the use of water as the dispersing medium do not possess the characteristic morphology and vital staining reactions exhibited by these structures in the intact liver cell. Our inability to find activity in such preparations supports the view of Hogeboom *et al.* that "large granules" obtained in this manner may have undergone irreversible structural changes due to the hypotonic conditions, which may have caused rupture or leaching out of necessary components of the enzyme system.

When homogenates of rat liver in isotonic saline solutions are fractionated according to the technique described by Claude (4), the distribution of activity in the oxidation of fatty acids is far different from that observed in fractions of 0.88 M sucrose homogenates. Data of such an experiment are given in Table VIII. It is seen that the fatty acid oxidase activity is concentrated in the "nuclear precipitate," containing nuclei, débris, and whole cells, and that the "large granule" fraction is without activity. It has been pointed out by Hogeboom *et al.* (6) that this first fraction of nuclei and débris may actually contain a large proportion of the mitochondria of the homogenate, since these particles are very largely agglutinated by contact with isotonic saline solutions and are therefore sedimented with the nuclear fraction. As a matter of fact, it is this fraction which has been used in this laboratory in the past for preparation of the fatty acid oxidase system. When this fraction is suspended in 0.88 M sucrose, a large part of the mitochondria is redispersed, and is sedimentable only at higher speeds, as the experiment in Table III indicates.

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[1] DNA, desoxypentose nucleic acid; PNA, pentose nucleic acid.

TABLE VIII

Distribution of Fatty Acid Oxidase Activity in Subcellular Fractions Derived from Isotonic Saline Homogenates of Rat Liver

The test conditions were the same as those described in Table I, except that the final volume was 3.0 ml. Incubation was for 30 minutes at 30°.

Fraction	Enzyme N per flask	Octanoate added	O_2 uptake	Acetoacetate formed
	mg.		μM	μM
"Large granules"	1.1	−	1.6	0.60
		+	1.25	0.31
"Nuclear ppt."	2.2	−	1.6	0.6
		+	11.0	3.4
Supernatant	2.3	−	0.0	0.60
		+	0.5	0.49

The fact that the less easily sedimentable fraction called "large granules" by Claude (4), obtained upon more prolonged centrifugation of saline homogenates in the experiment summarized in Table VIII, is inactive in the oxidation of fatty acids may possess some significance. Apparently the "large granules" derived from saline homogenates are not identical with those prepared by the hypertonic sucrose method, or they may be partially disorganized mitochondria of different sedimentation and enzymatic properties. Results of a similar nature reported by Chantrenne (23) lend some support to this view-point.

These findings point to the possibility that the fatty acid oxidase activity may be used as an enzymatic indicator for the detection of liver mitochondria in more or less the native condition, in conjunction with microscopic examination and staining techniques.

Intracellular Distribution of Some Glycolytic Enzymes—The finding that highly organized respiratory systems are localized in the mitochondria raises the question of the intracellular location of other organized enzyme systems. In contrast to the respiratory systems, which are associated with particulate matter, the glycolytic enzymes all appear to be readily soluble and several have been crystallized. It was therefore of interest to examine the different fractions of rat liver for their ability to catalyze the oxidation-reduction reactions of glycolysis. The system studied involved fructose-1,6-diphosphate as substrate, aldolase, triose phosphate dehydrogenase, diphosphopyridine nucleotide, and arsenate to "decouple" the phosphorylation step, and lactic dehydrogenase and pyruvate as hydrogen acceptors.

TABLE IX

Activity of Some Glycolytic Enzymes in Liver Fractions

In the oxidation-reduction system assay the Warburg vessels contained 0.03 M fructose-1,6-diphosphate, 0.002 M arsenate, 0.02 M sodium fluoride, 0.02 M sodium pyruvate, 0.048 M $NaHCO_3$, 0.02 M nicotinamide, and 0.001 M diphosphopyridine nucleotide. The liver fractions in amounts specified were tipped in from the side arm after temperature equilibration. Total volume, 2.0 ml.; gas phase, 95 per cent N_2-5 per cent CO_2; temperature, 30°; time of incubation, 1 hour.

Fraction	Weight of whole liver from which fraction was derived	CO_2 liberated	Oxidation-reduction activity*	Aldolase activity†
	mg.	*c.mm.*	*per cent of total*	*per cent of total*
Nuclear	60	18	5.4	3
Mitochondria	60	10	3.0	1
Supernatant	20	90	81.7	96
Whole homogenate	20	110	(100)	(100)

* The $Q_{CO_2}^{N_2}$ (30°) of the whole liver homogenate under these conditions was 27.5.

† Q_{HDP} (24) at 38° of whole liver homogenate was 56.

Fluoride was added to inhibit enolase and the end-point measured manometrically indicated the formation of 3-phosphoglyceric acid, which causes CO_2 liberation from a bicarbonate buffer. The conditions used were found to give approximately linear results with varying concentrations of an extract of rabbit muscle. When the different fractions were assayed for the presence of the three enzymes involved in the reaction, the mitochondria and the nuclear precipitate contained only a very small fraction of the activity, whereas the supernatant, containing all the soluble material of rat liver as well as difficultly sedimentable particles ("microsomes," etc.), contained 82 per cent of the activity shown by the original unfractionated homogenate (see Table IX). Also shown in Table IX is the distribution of aldolase in the different fractions, measured by the method of Sibley and Lehninger (24). It is seen that the particulate fractions contain only a very small fraction of the total aldolase of rat liver, 96 per cent being present in the soluble fraction.

Although assay of all the individual enzymes of glycolysis in these fractions may eventually show that one or more of these enzymes are present in the mitochondria, it appears certain that the mitochondria do not possess the complete enzymatic machinery for the conversion of glycogen or glucose to lactic acid at a rate comparable to the rate of respiration of these bodies. LePage and Schneider have recently shown that particulate fractions of rat tumors or rabbit liver have little or no ability to glycolyze glucose, most of the activity being in the soluble fraction (25).

In addition to these data, it has been found by Friedkin in this laboratory (*cf.* (26)) that isolated mitochondria are capable of incorporating inorganic phosphate labeled with P^{32} into pentose nucleic acid, phospholipide, and an unidentified acid-insoluble "phosphoprotein" fraction coupled to the oxidation of substrates of the Krebs tricarboxylic acid cycle. It would therefore appear that these bodies are also capable of at least one type of reaction leading to synthesis of these intracellular materials.

DISCUSSION

The data reported in this paper show that the complex enzyme systems responsible for the oxidation of fatty acids and Krebs cycle intermediates and esterification of phosphate coupled to these oxidations are localized in that fraction of rat liver which consists of morphologically intact mitochondria or "large granules," almost completely free of other formed elements. As has been pointed out, it is not possible with our present knowledge of these complex systems to assay individual enzymes of these systems quantitatively and it is therefore conceivable that other elements such as the nuclei, "microsomes," or soluble material may be capable of many of the enzymatic transformations involved in the over-all reactions studied. The striking fact is that all the individual enzymes concerned in these complex systems should be found in one species of morphological element. These findings in some measure justify the early views of Altmann (27) that these bodies are fundamental biological units and possess a certain degree of autonomy and certainly, together with the considerable work already done on their enzymatic and chemical composition by the Rockefeller school, Schneider, and others, provide considerable basis for the apt designation "intracellular power plants" conferred on the mitochondria by Claude.

Although the mitochondria appear to be the major site of these activities, it would appear from our examination *in vitro* that these bodies are not completely autonomous with respect to their respiratory behavior, since they must be supplemented with certain cofactors such as adenosine triphosphate and Mg^{++}. It appears likely that in the cell there is a rapid interchange of these factors, substrates, and inorganic phosphate between the cytoplasm and the mitochondria. It also would appear that these bodies are dependent on the cytoplasm for certain preparatory metabolic activities such as glycolysis, since, as our data show, they are almost completely lacking in glycolytic activity.

Claude has found that isolated mitochondria are quite sensitive to changes in osmotic pressure (4). Adverse osmotic conditions may therefore be responsible for the inactivity of the mitochondria in catalyzing fatty acid oxidation in hypotonic reaction media; when the concentration of neutral salts or non-electrolytes approximates isotonicity, the system shows maximum activity. No attempts have been made to determine whether the mitochondria are morphologically intact in all stages of the enzymatic reaction.

It is also of some interest that mitochondria are capable of causing oxidation-coupled incorporation of labeled inorganic phosphate into nucleic acids and phospholipides of these structures (*cf.* (26)). The work of Hill and Scarisbrick (28) and Warburg and Lüttgens (29) on the photochemical activity of isolated chloroplasts or granules derived therefrom provides some indication that highly organized enzyme systems are localized in analogous structures of plant cells.

The localization of organized respiratory activity in mitochondria poses some problems in connection with the separation and purification of the individual enzymes involved. The difficulties in rendering such enzymes as cytochrome oxidase and succinoxidase soluble are well known (30, 31). Recent work in this laboratory indicates that the separation from mitochondrial preparations in soluble form of simple dehydrogenase proteins which might be expected to be readily soluble is also quite difficult and a variety of drastic procedures has failed to release any significant amount of such proteins into soluble form.

SUMMARY

Morphologically homogeneous mitochondria ("large granules") separated from rat liver dispersions by the hypertonic sucrose method of Hogeboom, Schneider, and Pallade contain essentially all the measurable activity of the liver in the oxidation of fatty acids. Likewise, the integrated reactions of the Krebs tricarboxylic acid cycle are found in this fraction. Esterification of inorganic phosphate accompanies these oxidations in purified preparations of mitochondria. These bodies have insignificant glycolytic activity. "Mitochondria" prepared by other methods involving saline or water as the dispersing media are inactive in these reactions, possibly because of osmotic damage.

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SUBSTRATE UTILIZATION IN MAMMALIAN ERYTHROCYTES

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There has been frequent reference to the formation of carbon dioxide during red blood cell metabolism. Phenylhydrazine treatment of erythrocytes, by supplying an autoxidizable component, hemin, was said by Warburg to stimulate carbohydrate utilization and carbon dioxide production (1). The presence of either methylene blue or methemoglobin (MHb) was found to increase erythrocyte respiration, giving an R.Q. which was less than 1.0 because of pyruvate formation (2). Carbohydrate balance studies in which it was demonstrated that more glucose disappeared from methylene blue-treated red cells than could be accounted for as lactate and pyruvate led Wendel to the conclusion that glucose is metabolized by two paths, one leading to pyruvate, the other to carbon dioxide (3). Acetaldehyde has been identified in erythrocytes by Barker (4), but according to Wendel it is not formed from pyruvate, the latter being inert in red cells (3). Although red corpuscles contain cocarboxylase (5), no specific reaction of decarboxylation has been identified in them. It seemed probable that malate and fumarate, which are known to be utilized by erythrocytes (6) would serve as substrate for such a reaction. This question was studied in manometric experiments after it was first determined that lactic acid is formed from these substrates in washed erythrocytes.

The metabolism of four sugars in red cells, investigated recently in regard to their ability to reduce methemoglobin (6), is studied here in connection with glycolysis, in part at least to compare these two reactions. In addition, the manometrically determined reaction rates with the several substrates and MHb reduction rates are compared for three species, dog, cat, and rabbit.

EXPERIMENTAL

Measurement of Lactic Acid—Lactate was determined in erythrocytes obtained from the pooled blood samples of several rabbits, washed six times by centrifugation in about 6 volumes of sterile physiological saline. The packed cells were resuspended in an equal volume of a 1:1 solution of 0.85 per cent NaCl and 0.1 M Na_2HPO_4 brought to pH 7.4 with 1 N

HCl (saline-phosphate buffer). To 5.0 ml. of the suspension placed in each of four paraffinized flasks was added 0.7 ml. of 0.85 per cent saline for a control, or 0.7 ml. of 0.25 M solution of sodium malate (Eastman), fumarate (Eastman practical, recrystallized), or succinate (Eastman). A sample was removed for lactic acid determination (7) immediately upon addition of each substrate and at intervals during incubation in a 38° water bath. This experiment showed an increase in lactic acid in washed erythrocytes incubated with malate or fumarate, but not in cells treated with succinate (Table I). Also apparent in Table I is the fact that after 2 hours fumarate is more effective than malate in lactic acid formation. Lactate formation from malate was confirmed with dog and cat erythrocytes. The inability of hemolyzed cells to produce lactic acid from added malate or fumarate was demonstrated in a similar experiment on washed cells diluted 1:3 in distilled water.

TABLE I

Lactic Acid Formation in Rabbit Erythrocytes

The results are expressed in mg. per 100 ml. of cell suspension.

Substrate	Hrs. after adding substrate			
	0	2	4	6
Control*	5.5	5.6	11.8	10.2
Malate*	13.1	29.1	40.8	53.7
Fumarate*	5.1	26.0	51.0	62.6
Succinate	4.2	6.3	13.1	9.0

* Average results from four experiments with pooled blood of several animals.

Evidence was available from results in Table I, and the experiment on hemolyzed cells, that malate interferes in the lactic acid determination. This point was investigated by using the chosen method for lactic acid measurement on pure water solutions of malate and fumarate. Values of 0.0 and 111.0 γ of lactic acid were obtained on 0.1 ml. (3.3 mg.) of 0.25 M fumarate and malate, respectively. The interference was not determined with the malic acid later purified for use in the manometric tests.

Manometric Studies—As a more accurate and convenient means of measuring the rate of substrate utilization in washed erythrocytes, the anaerobic Warburg method was employed (8). For these runs heparinized[1] blood samples from a number of animals of a species were pooled and washed four to six times as described. Finally the cells were centrifuged 30 minutes at 1700 × g and stored overnight in the cold. A measured volume of the packed cells was mixed with an equal volume of Krebs-Ringer bicarbonate buffer (8), and 4.0 ml. of the cell suspension were placed in the main chamber of a 15 ml. capacity Warburg flask. In the tests employing metabolic intermediates 0.7 ml. of 0.25 M substrate solution was placed in the flask side arm; in hexose runs, 0.3 ml. of sugar solution was used with Krebs-Ringer bicarbonate as needed to equalize dilution in all vessels. Fructose was used in 10 per cent and the other sugars in 5 per cent solution. Substrate-free control vessels (endogenous controls) received 4.0 ml. of cell suspension in the main chamber and 0.7 ml. of Krebs-Ringer bicarbonate in the side arm. The flasks were fitted into the 40° water bath and an anaerobic system was achieved by 10 minutes ventilation with 5 per cent carbon dioxide in nitrogen, first passed through a hot copper furnace. After gas liberation had ceased or reached a slow even rate in all flasks, the substrates were tipped into the main chamber. Readings were taken at intervals of 15 or 20 minutes.

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[1] Purified Connaught heparin was used, since crude heparin inhibited hexose utilization.

TABLE II

CO_2 Evolution by MHb and MHb-Free Rabbit Erythrocytes from Various Substrates

The results are expressed in microliters per hour.

Substrate	Experiment 1		Experiment 2		Experiment 3	
	MHb	No MHb	MHb	No MHb	MHb	No MHb
Malate	45.2	30.6	*	30.6	36.6	27.8
Fumarate	64.3	41.7	58.8	36.1	55.5	38.1
Glucose	*	40.5	15.1	26.5	24.5	43.8
Control	17.0	7.4	11.0	4.0	5.3	2.6
Lactate	12.6	2.8	13.1	0.0	−5.5	−4.8
Pyruvate	26.3	14.0	11.7	11.7	9.6	10.5
Succinate					−3.6	2.9

Blood cells were prepared as follows: Experiments 1 and 2, 0.14 ml. of 1 per cent $NaNO_2$ per ml. of cells was added to half the cells (washed six times) at room temperature 2 hours before the rates were measured. Experiment 3, half the sample was incubated at 38° for 30 minutes with 0.14 ml. of 1 per cent $NaNO_2$ per ml. of cells before washing six times; non-MHb cells were incubated 30 minutes at 38° and washed simultaneously with MHb cells. The rates are given for the period 1½ to 3½ hours after tipping in the substrates.

* Run failed through technical fault.

Carbon dioxide evolution was observed in washed erythrocytes when supplemented with glucose, fructose, mannose, galactose, fumarate, malate, or pyruvate, but not when supplemented with lactate or succinate (Tables II and III). No gas was evolved in the absence of red cells with any of these substrates tipped into Krebs-Ringer bicarbonate buffer, except for an unsustained expulsion of gas with pyruvate which stopped completely within 75 minutes after tipping. Malic and fumaric acids did not promote carbon dioxide evolution when added to a 1:1.5 or 1:3 dilution of red corpuscles in water with or without additional Krebs-Ringer bicarbonate. Though rates varied considerably from day to day, especially with cat cells, the average per cent deviation from the mean for duplicate flasks in thirty-nine runs was 3.8. Steady carbon dioxide liberation occurred with malate and fumarate when the bicarbonate medium was replaced by the saline-phosphate buffer.

TABLE III

Summary of Tests from Which Course of Substrate Utilization (Figs. 1 to 3) Was Determined

The results are expressed in microliters per hour.

Substrate	Dogs			Cats			Rabbits		
	No. of runs*	Average rate†	Standard deviation	No. of runs*	Average rate†	Standard deviation	No. of runs*	Average rate†	Standard deviation
Glucose	27	21.0	3.6	5	25.8	7.0	13	48.7	10.4
Fructose	25	20.5	4.8	5	24.5	11.6	6	59.3	11.7
Mannose	25	22.0	4.0	5	19.6	7.6	6	56.8	12.3
Galactose	6	1.2	0.9	3	−0.5	1.8	3	13.5	3.1
Endogenous control	6	−3.8	1.9	6	−2.6	1.8	3	3.5	2.0
Pyruvate‡	3	4.0	4.9	4	−0.4	2.2	2	11.1	8.1
Malate	5	17.4	6.6	5	4.3	3.6	7	38.3	7.2
Fumarate	6	33.8	7.5	6	8.9	4.0	7	44.5	7.7

* Erythrocytes used for each run were derived from pooled blood samples from six to fifteen (usually twelve) animals in each species.

† The figures represent averages of rates computed for the period 2 to $3\frac{2}{3}$ hours after tipping, except for hexose rates in rabbits, which were computed for 1 to $3\frac{2}{3}$ hours.

‡ Rate corrected for gas evolved by substrate blank.

Table II shows that carbon dioxide liberation is more rapid with malate and fumarate and less rapid with glucose in methemoglobinized than in methemoglobin-free corpuscles. It is also apparent that lactate and succinate are not utilized.

In Table III are shown the number of runs made and the average rates of carbon dioxide liberation obtained in washed dog, cat, or rabbit erythrocytes supplemented with any of seven active substrates or treated with buffer alone. Rates at different phases of the run obtained by averaging results from the several trials are shown in Figs. 1 to 3.

It is to be noted that in dog cells the rate obtained with glucose, fructose, or mannose increased progressively throughout the run. Fructose in this species, in contrast to the other hexoses, caused gas absorption during the 1st hour and then gave a steadily increasing rate of carbon

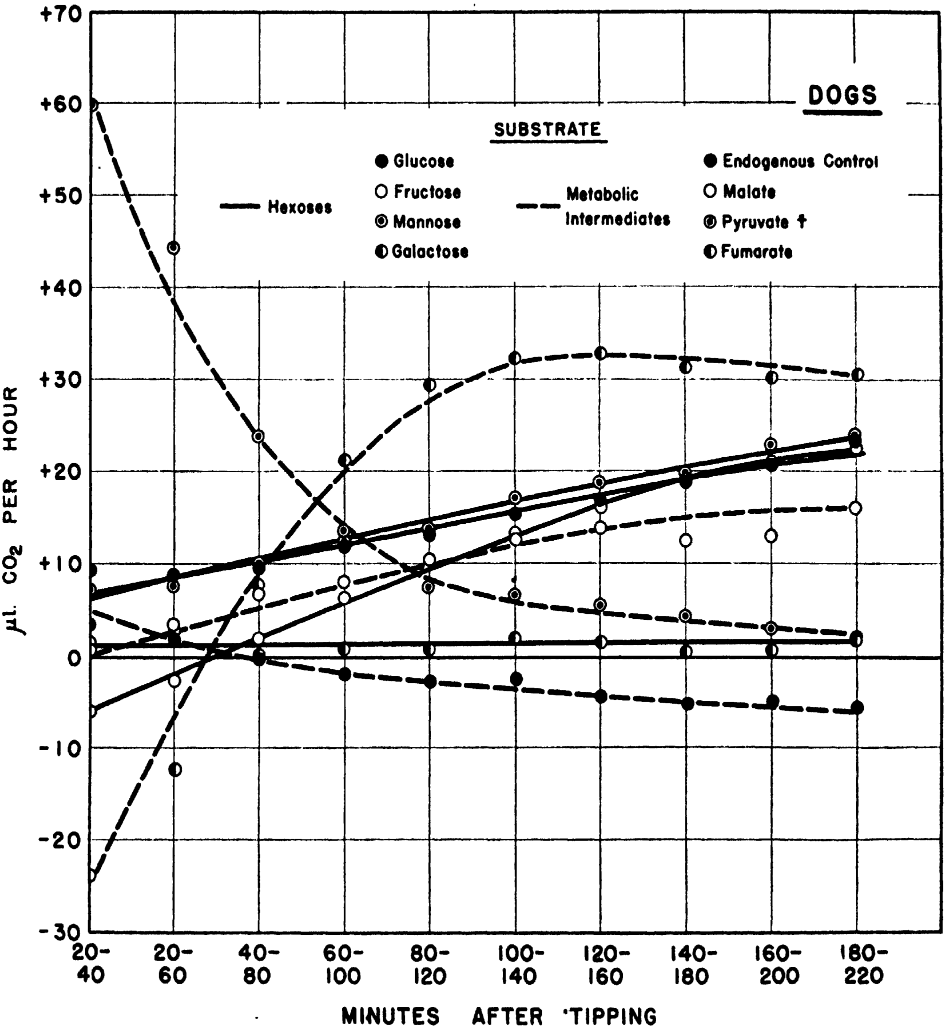

FIG. 1. Carbon dioxide evolution at intervals in the course of substrate utilization in washed dog erythrocytes. †, corrected for substrate blank.

dioxide liberation which after 3 hours equaled that of glucose and mannose. Since a period of absorption invariably preceded the phase of gas evolution in all fructose runs in dogs whether 0.3 or 0.6 ml. of 5 per cent or 0.3 ml. of 10 per cent solution was employed, this initial negative phase was not due to a possible hypertonic effect of the 10 per cent solution. Moreover, with either glucose or mannose as substrate in dog cells no difference in any phase of the run was noted whether 0.3 ml. of 10 per cent or 0.3 or 0.6 ml. of 5 per cent solution was used. The period of absorption with fructose occurred at a time when a small amount of gas was still evolving from endogenous controls.

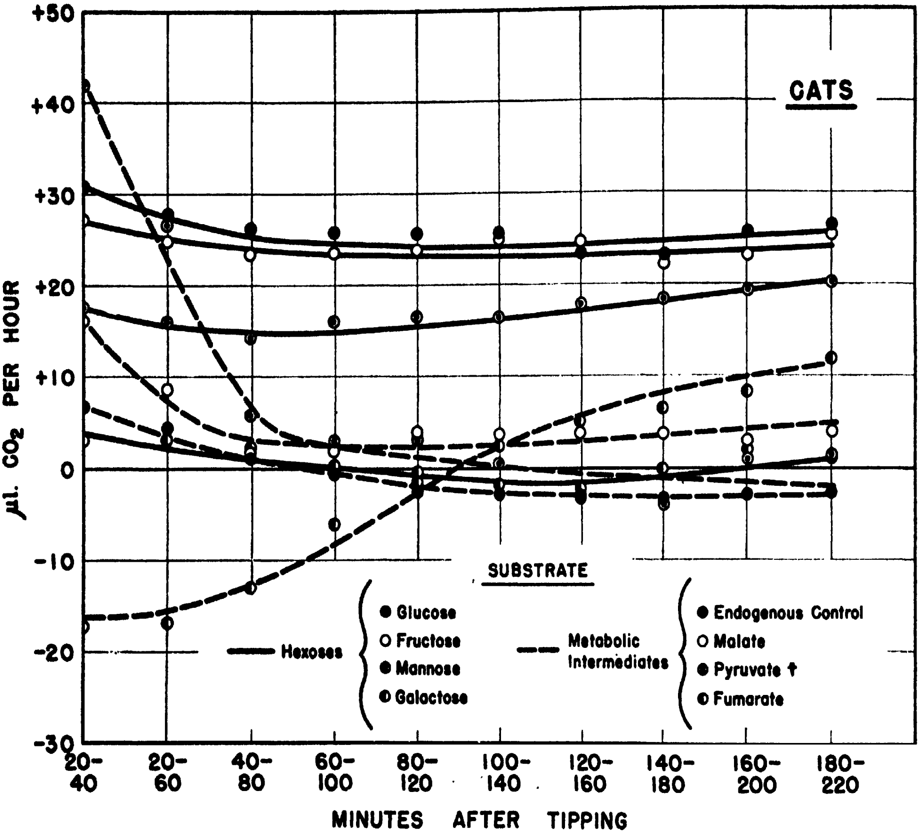

FIG. 2. Course of substrate utilization in cat erythrocytes. †, corrected for substrate blank.

The metabolism of these hexoses in cat erythrocytes was relatively steady from the beginning, being somewhat slower with mannose than with the other two (Fig. 2). In this species the average rates given for hexose were derived only from runs in which glucose, fructose, and mannose were employed simultaneously on a portion of a cell suspension.

As shown in Fig. 3 a steady rate of utilization was obtained also in rabbit cells, except that fructose and mannose failed to attain a maximal rate as quickly as glucose. Fructose and mannose invariably gave higher rates than glucose each of five times these three hexoses were tested simultaneously with aliquots of a batch of rabbit cells. There is a tend-

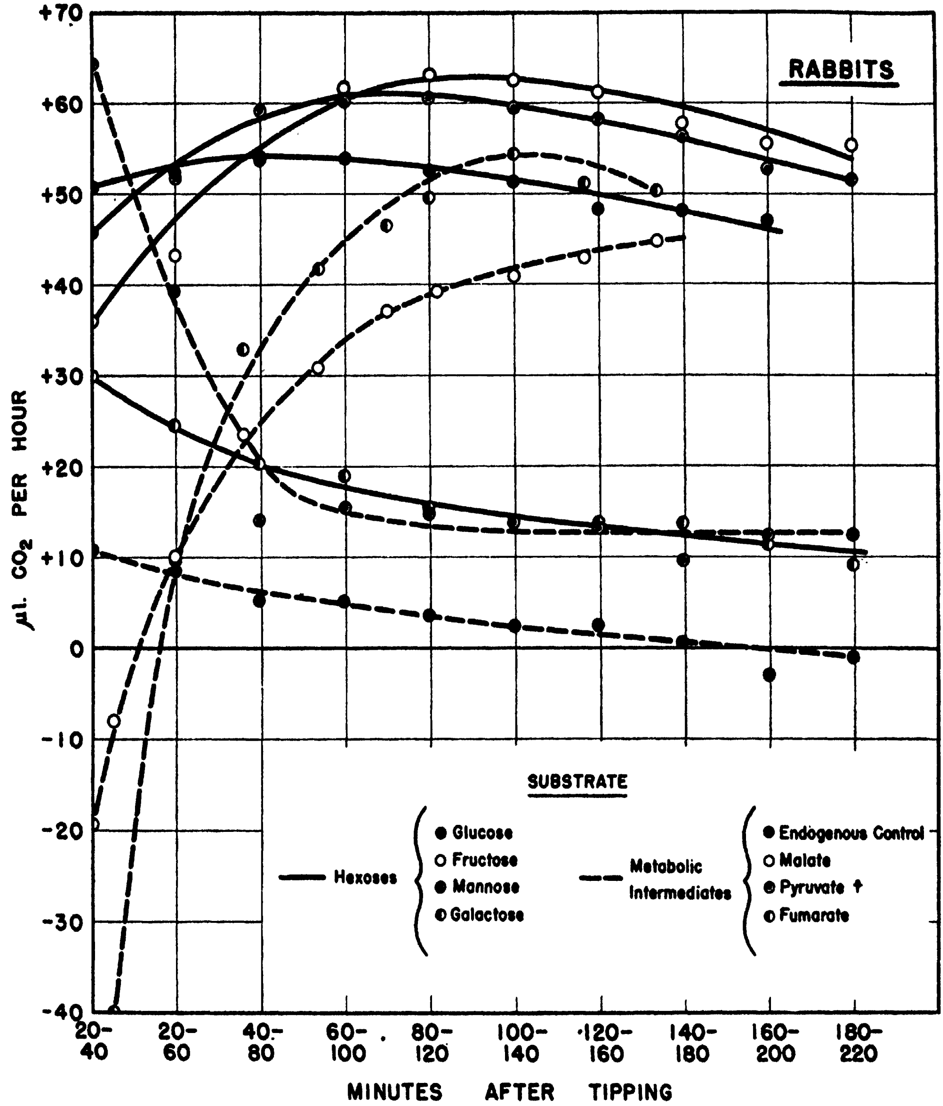

FIG. 3. Course of substrate utilization in rabbit erythrocytes. †, corrected for substrate blank.

ency for the rate of hexose utilization to drop off after 2 hours in rabbit corpuscles; this trend might be due to greater lability of the very active rabbit glycolytic mechanism.

Galactose caused slow gas evolution compared with the other sugars. Each of the sugars gave approximately the same rate in cats as in dogs but a rate almost 2 times greater in rabbits.

A wide variation in results was obtained among the three species in the experiments with metabolic intermediates. The carbon dioxide liberation by dog erythrocytes supplemented with malate began at a low rate which increased progressively for the duration of the experiment. This picture was reversed in cat cells, in which gas evolution started at a relatively high level and decreased until a slow, steady rate was reached after about an hour. Gas absorption in the early stages of the run with malate in rabbit erythrocytes gave way to rather rapid evolution as the experiment progressed, the rate leveling off after about 2 hours.

Fumarate, following an early period of gas absorption, finally produced a constant rate of evolution in dogs and rabbits; the period of absorption was too prolonged in cats to make it possible to determine whether a constant rate with fumarate would have been reached eventually. Pyruvate utilization is apparent from the slow but persistent rate obtained over a period of 3½ hours or more. This rate, computed from 2 hours after tipping substrate, was preceded by an unexplained expulsion of gas at the outset which continued for the 1st hour after tipping.

The rates at which the several intermediates are utilized are several times greater in dogs than in cats, and nearly two times greater in rabbits than in dogs. Control vessels show a small and gradually vanishing carbon dioxide output, which is most marked and prolonged in rabbits, and which might be associated with the presence of impermeable hexose esters not removed during washing. This was the explanation given by Wendel for the lactate formation in unsupplemented washed dog corpuscles (3). The greater activity of rabbit endogenous controls may be compared with the ability of unsupplemented washed rabbit cells to reduce MHb, in contrast to the absence of such reduction in similarly prepared cat and dog cells.[3]

The effect of three hexoses added at various levels to washed dog and rabbit erythrocytes is shown in Table IV. The three sugars were used with aliquots of the same batch of cells at each level tested. It is apparent that with glucose and mannose a maximal rate of utilization is approached in both species by increasing the concentration of substrate to about 300 mg. per cent. Fructose, on the other hand, is much less effective than glucose or mannose at low levels but yields greater rates than these when added in high concentrations. The relative ability of these three sugars at varying concentrations to produce carbon dioxide evolution in dog erythrocytes corresponds closely with their relative ability to reduce methemoglobin (6).

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[3] Unpublished results.

The effect of the sugars in combination was determined with dog erythrocytes. In Table V are given the rates of carbon dioxide liberation in several experiments in which aliquots of a washed red cell suspension were tested with hexoses singly or in paired combinations. To 4.0 ml. of cell suspension prepared from the washed pooled blood of six dogs were added 0.3 ml. of the 5 per cent sugar solutions (except fructose, 10 per cent) and Krebs-Ringer bicarbonate buffer to equalize dilutions. Galactose alone, it appears, is additive in effect with the other hexoses. This finding and the lowered effectiveness of mannose and fructose in combination were also observed in utilization of the sugars to reduce MHb (6).

TABLE IV

Rate of Carbon Dioxide Evolution (Microliters per Hour) by Dog and Rabbit Erythrocytes in Relation to Hexose Concentration*

Substrate	Glucose		Fructose		Mannose	
	Dogs	Rabbits	Dogs	Rabbits	Dogs	Rabbits
mg. per cent						
14.7	8.7		−5.1		10.1	
29.4	15.9		−4.3		16.4	
58.7	17.6		−1.7		17.0	
146.8	18.5		1.6		17.8	
292.6	20.0	50.6	14.5		23.6	54.1
586.5	21.7	49.8	22.9	59.7	20.6	49.0
1174.5			30.7	67.7		

* The rates were computed for the period 2 to 3½ hours after tipping.

An initial burst of gas evolution at tipping substrate or Krebs-Ringer bicarbonate buffer into the red cell suspension was noted in all the manometric tests. This sudden gas expulsion in hexose runs appears to be due mainly to the effect of the buffer in the side arm, since the magnitude of the burst decreases in order with buffer alone, mixed solutions of equal quantities of buffer and sugar, and sugar alone. Although the burst was somewhat larger with fructose than with other sugars, the characteristic early period of absorption in fructose flasks apparently is not a compensation for the large burst at tipping, since fructose in combination with other sugars caused the usual initial negative period without a larger burst.

Fumarate has invariably proved superior to malate in our experiments. In order to rule out the possibility that the lesser activity of malate might be due either to inhibitory contaminants in our malate preparation or to inhibition of its metabolism by excess malate, experiments were conducted at several levels with 98 per cent pure malic acid[3] (9) and recrystallized fumaric acid. The results in Table VI show the advantage of

TABLE V

Additive Effect of Hexoses in Dog Erythrocytes

All rates are given as microliters of CO_2 per hour for the period 2 to 4 hours after tipping. The per cent change was computed from the greater of the two single hexose rates involved.

Substrate*	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5		Experiment 6	
	Rate	Per cent change	Rate	Per cent change	Rate	Per cent change	Rate	Per cent change	Rate	Per cent change	Rate	Per cent change
	µl.		µl.		µl.		µl.		µl.		µl.	
G.	23.5		25.4		12.6		26.0		21.0†		25.3	
F.	24.1		20.4		10.3†		30.4		18.5		30.5	
M.	27.0		26.6		15.2		25.8†		18.7†		26.2†	
Ga.	2.2		0.0		1.4		0.9					
G. + F.	23.0	−4.8	20.8	−22.1	11.9	−5.9	36.4	19.7	22.9	9.0	30.8†	1.0
" + M.	25.3	−6.7	20.3	−31.0	16.6	9.2	26.0	0.0	23.0†	9.5	28.8	9.9
M. + F.	22.0	−22.7	24.2	−9.9	16.0	5.3	25.3	−20.2	19.6†	4.8	24.0†	−27.1
G. + Ga.	26.8	14.0	28.7	13.0	16.3	29.4	30.2	16.2				
F. + "	28.2	17.0	29.2	43.1	18.0	74.8	33.8	11.2				
M. + "	28.4	5.2	30.3	13.9	19.1	25.6						

Substrate*	Experiment 7		Experiment 8		Experiment 9		Experiment 10		Average per cent change with combinations
	Rate	Per cent change	Rate	Per cent change	Rate	Per cent change	Rate	Per cent change	
	µl.		µl.		µl.		µl.		
G.	14.8†		18.8†		18.7†		23.2†		
F.	24.9		19.6†		21.0†		22.4†		
M.	14.6†		23.8†		23.0†		26.1		
Ga.									
G. + F.	25.6†	2.8							0.0
" + M.	17.3	16.9							1.1
M. + F.	19.3†	−29.0							−14.1
G. + Ga.			22.4†	19.1	23.2†	24.1	25.9†	11.6	18.2
F. + "			27.2†	38.8	26.5†	26.2	32.3†	44.2	36.5
M. + "			24.6†	3.4	23.8†	3.5	27.2†	4.2	9.3

* G. represents glucose; F., fructose; M., mannose; and Ga., galactose.

† Represents average of results obtained on duplicate flasks.

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[3] Melting point, 98–98.5°; specific rotation: acetone, $c = 4.76$, $[\alpha]_D^{20} = -6.7° \pm 0.1°$. The method of purification was modified at the point of final recrystallization, which was effected from ether solution with benzene.

fumarate over malate at the several levels tested with these preparations. The same ratio of fumarate to malate activity was obtained with these substrate and cell preparations when a portion of the cells was converted to "MHb cells" (6) and the rate of MHb reduction was determined, except that at the highest concentration fumarate and malate were equally effective.

MHb reduction rates in methemoglobinized washed erythrocytes of the three species were measured by using the four hexoses and sodium lactate, malate, and fumarate as substrates according to the method described previously (6). Comparing manometric and MHb reduction rates shows but little disparity between the two means of studying red cell metabolism. In both types of measurements rabbits gave the fastest rates with all substrates and cats the slowest, except that cats and dogs have the same rates with sugars. The order of utilization of the hexoses showed one definite discrepancy between MHb reduction and manometric rates in that in dogs mannose was relatively slower than glucose and fructose only in regard to MHb reduction.

TABLE VI

Rate of Carbon Dioxide Evolution in Rabbit Erythrocytes according to Concentration of Malate or Fumarate[*]

Ml. of 0.25 M substrate added per 4 ml. cell suspension	Malate	Fumarate
	μl. CO_2 per hr.	*μl. CO_2 per hr.*
0.03	−0.6	5.1
0.05	0.9	
0.09	3.6	12.7
0.18	9.0	20.2
0.36	14.8	27.8
0.70	27.6	35.2

[*] Similar results were obtained with commercial C.P. malic and fumaric acids (Bios).

On plotting the MHb concentration against time two types of curves were encountered: one giving a straight line on log paper was obtained with all seven substrates in rabbit cells and with lactate, malate, and fumarate in dog or cat cells, and the other giving a straight line on a linear plot was obtained with the hexoses in cat and dog cells. Fumarate, and less definitely malate, showed a lag period of about 30 minutes before a maximal rate of MHb reduction was reached. This was most distinct in rabbit MHb corpuscles, for which the rates attained were relatively very rapid.

DISCUSSION

The malate conversion to lactate in erythrocytes in all probability resembles anaerobic glycolytic lactate formation in its alternate oxidation and reduction of di- or triphosphopyridine nucleotide. Acceleration of the rate of gas evolution with malate or fumarate by the presence in the cell of MHb would appear to be due to oxidation of and increase in the rate of turnover of the pyridine coenzyme. The supply of the oxidized coenzyme may then perhaps be regarded as a limiting factor in determining the rate. The impaired glycolysis in MHb-containing cells may be due either to an effect of MHb on glucose metabolism or, more likely, to an unfavorable effect of nitrite on the glycolytic mechanism.

An explanation for the superiority of fumarate over malate could be either that the erythrocyte is more permeable to fumarate or that it metabolizes fumarate by a route other than through malate. The steady gas evolution with malate and fumarate in either saline-phosphate or bicarbonate buffer probably results from decarboxylation. The lactate determinations establish that such a reaction does occur in erythrocytes. That a slow rate is obtained manometrically with large amounts of pyruvate suggests utilization of this substrate in a limited manner in some reaction such as decarboxylation.

The relative effectiveness of glucose, fructose, and mannose might be explained on a basis of affinity for an enzyme required in common, such as hexokinase. This could explain also the failure of these sugars to improve one another additively. The relative order found for the rate of phosphorylation of fructose, glucose, and mannose by yeast hexokinase, *i.e.* 1.4:1.0:0.3 (10), is not in good agreement with the relative rate of utilization found here in erythrocytes. According to Krahl and Cori, the enzyme hexokinase probably limits the rate of glucose utilization in rat diaphragm (11). A delay in utilization of fructose could be explained, perhaps, by postulating a path for fructose metabolism in red cells similar to that thought to occur in liver (12), *i.e.* via fructose-1-phosphate. It is not understood why a preliminary phase of gas absorption occurs in dog cells with this substrate. Transfer of phosphate from adenosine triphosphate to glucose, the initial step in glucose metabolism, involves liberation of 1 acid equivalent (10). By way of explanation of the progressively increasing rates with hexoses in dogs, it may be suggested that the increase in pyrophosphate on incubation of blood (13), *i.e.* the progressive increase in adenosine triphosphate resulting from anaerobic glycolysis (14), increases the rate of utilization of the hexoses in a progressive manner.

The fact that rates are faster in rabbit cells than in cat and dog corpuscles with each substrate might be a result of greater permeability of rabbit erythrocytes or it might be due to greater concentration or rate of turnover of the essential respiratory enzymes. A similar explanation may be projected for the feeble utilization of the intermediates in cat cells. Even the endogenous controls are faster in rabbits, and this activity presumably is due to impermeable metabolites retained in the washed cell.

SUMMARY

The formation of lactic acid from metabolic intermediates in washed erythrocytes has been investigated. Malate and fumarate, but not succinate, are converted to lactic acid in rabbit erythrocytes. Commercial malic acid interferes with the determination of lactic acid by the method used. Conversion of malate or fumarate to lactic acid was not observed in hemolysates.

The metabolism of seven substrates has been studied manometrically in erythrocytes of three species. Red blood cells treated with either malate or fumarate evolve carbon dioxide anaerobically from bicarbonate medium and do so more rapidly in the presence of methemoglobin. Fumarate metabolism, after an initial negative phase, proceeds at a faster rate than malate. Pyruvate produces a slow but persistent rate of gas evolution in these anaerobic tests.

Four hexoses are metabolized in dog, cat, and rabbit erythrocytes, the relative activity being approximately the same for mannose, glucose, and fructose, but much less for galactose. Fructose reaches a maximal rate more slowly than glucose and mannose in rabbit and dog corpuscles, and in the latter shows an initial period of gas absorption whether used singly or in paired combination with the other sugars. Glucose, fructose, and mannose yield progressively increasing rates in dogs, and steady rates in cats and rabbits. Fructose in dog erythrocytes is less active than glucose and mannose at low, and more active at high, concentrations. Galactose activity is additive with that of the other sugars in dog corpuscles; fructose and mannose combined are less effective than the faster of these sugars used alone.

Metabolic activity with hexoses in rabbit cells is nearly double that in cat and dog cells. With malate, fumarate, and pyruvate, carbon dioxide evolution is accomplished nearly twice as fast in rabbits as in dogs, which in turn utilize these substrates several times faster than cats. Succinate and lactate cause no gas liberation in the erythrocytes of any of the three species.

MHb reduction rates agree in general with manometrically determined rates in regard to relative effectiveness of the substrates and activity in the three species. MHb reduction follows the pattern of a first order reaction except for resembling a zero order reaction with hexoses in dog and cat cells.

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THE INCORPORATION OF RADIOACTIVE CARBON DIOXIDE AND ACETATE INTO LIVER PROTEINS IN VITRO*

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During the past 2 years, a number of papers have appeared dealing with the subject of *in vitro* peptide bond synthesis. Melchior and Tarver (1) have described the incorporation of radioactive methionine into the proteins of rat liver slices. From this laboratory a preliminary report (2) has been presented describing the results of experiments on the fixation of radioactive carbon dioxide in rabbit liver slices in which the proteins of these slices were shown to contain dicarboxylic amino acids labeled in the carboxyl positions adjacent to the amino groups. Several other laboratories have reported the *in vitro* incorporation of C^{14}-labeled amino acids into the proteins and peptides of tissue slices (3–6), and homogenates (5, 7), and of erythrocytes (8). Cohen and McGilvery (9) have studied the formation of the peptide, *p*-aminohippuric acid, by liver homogenates and by the insoluble protein fraction obtained from these.

The significance of all of these studies (excepting those of Cohen and McGilvery in which a single, easily identifiable peptide compound was under consideration) depends on the assumption that the "turnover" actually takes place in the peptide chain of proteins. Other, but unlikely, possibilities are peptide bond formation on side chain amino and carboxyl groups, or the adsorption of added labeled compounds, not removed by the precipitating and washing procedures. Indeed, such possibilities would also apply to the bulk of the *in vivo* studies on which the theory of the dynamic state of body proteins stands.

Assuming, however, the unlikelihood of these possibilities, the data in the above reports lead to certain tentative conclusions: First, the necessity for oxidative energy; and second, the localization of at least some of the enzymes involved in the insoluble particles of the tissue cells. Whether or not the mechanism of incorporation of the amino acid units into the protein molecule is the same in those studies with labeled amino acids and in the present experiments in which the slices must synthesize their own radioactive molecules by CO_2 or acetate fixation is not clear at present. As will be seen below, certain experiments suggest the possibility that some radioactive compound other than the amino acid itself may be the immediate precursor to peptide bond turnover in the case of aspartic and glutamic acids.

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The *in situ* formation of labeled amino acids in the "turnover" studies to be reported below possesses one inherent advantage over *in vitro* experiments with labeled amino acids. The conditions under which protein turnover might take place are more nearly physiological than in the presence of an abnormal excess of a single labeled amino acid, ordinarily present in rather small amounts.

In the present paper, a comparison is made of the rates of incorporation in proteins of radioactivity from inorganic carbon dioxide and from carboxyl-tagged acetate. The results show that the carboxyl carbon of acetate is incorporated in the protein dicarboxylic amino acids without first becoming carbon dioxide. This acetate carboxyl carbon appears to be incorporated at a rate about one-quarter to one-half that of carbon dioxide incorporation.

Methods

Liver slices from fasted rabbits were incubated in suitable media containing radioactive carbon as $NaHC^{14}O_3$ or as carboxyl-labeled sodium acetate. The sodium acetate was prepared by a modification (10) of the method of Sakami, Evans, and Gurin (11). Except for the addition of 40 mM of pyruvate and 7.5 mM of alanine in most of the experiments, the incubation media were identical with those described previously (12).

Immediately after the removal of the liver from the animal, three slices, 0.5 mm. thick, were cut with the Stadie slicer and placed in 50 ml. Erlenmeyer flasks containing 3 ml. of incubation medium. The Erlenmeyer flasks had sealed in side arms, fitted with vaccine ports for adding reagents and removing gas samples for analysis. The mouths of the flasks carried 2-holed rubber stoppers through which passed two glass tubes to permit flushing the flasks with appropriate gas mixtures (5 per cent CO_2-95 per cent oxygen, unless otherwise stated). After a 5 minute period during which the gas mixture was passed through the vessels, the gassing ports were clamped shut, and an 0.04 to 0.08 ml. aliquot of $Na_2C^{14}O_3$ (about 0.0015 mM) or carboxyl-labeled sodium acetate (about 0.0017 mM) was added with a constriction pipette (13). These additions were made through the side tube and the vaccine ports which had been removed were quickly replaced. The vessels were rocked slowly in a constant temperature room at 38 ± 1°. At the end of the incubation period, 3 ml. of 10 per cent trichloroacetic acid were added through the vaccine port by means of a hypodermic syringe.

Gas samples were withdrawn with a syringe from the flask through the vaccine port and transferred at once to a centrifuge tube containing 1 ml. of barium hydroxide. The radioactivity of a weighed portion of the precipitated barium carbonate was measured and the specific activity of the inorganic carbon in the flask was calculated. Experiments showed that, with the ratio of medium to tissue employed, the specific activity of the inorganic carbon in vessels with added $Na_2C^{14}O_3$ did not change by more than 5 per cent during a 4 hour period of incubation.

The tissue slices and medium were transferred to a mortar and ground thoroughly. The finely ground tissue was then washed three times by centrifugation and resuspension in cold 5 per cent trichloroacetic acid, three times in 50 per cent alcohol-ether, and once in ether. The protein material remaining, after drying, was hydrolyzed for 20 hours in 6 N HCl. The hydrolysate was decolorized with charcoal, concentrated to dryness *in vacuo* over sodium hydroxide pellets, and made up to a known volume, usually 2 or 3 ml.

The dicarboxylic amino acid fraction was prepared from a portion of this hydrolysate. 1 ml. of the hydrolysate was brought to pH 10 with saturated barium hydroxide, and 0.2 ml. of additional $Ba(OH)_2$ was added to provide an excess. The addition of 3 volumes of alcohol gave a flocculent precipitate which was centrifuged. This barium precipitate was decomposed with sulfuric acid, the barium sulfate centrifuged out, and the dicarboxylic amino acids reprecipitated from the supernatant in the same manner as described above. After decomposition of this precipitate with sulfuric acid, the absence of appreciable contamination by other amino acids was demonstrated by the filter paper chromatograph technique of Consden, Gordon, and Martin (14). As a standard for comparison with the unknown solutions, a drop containing glutamic and aspartic acids in equal proportions plus phenylalanine to the extent of 5 per cent of one of these was placed on the filter paper strip. This amount of phenylalanine gave an easily detectable spot, while the unknown solutions yielded only the spots characteristic for glutamic and aspartic acids. The purity of the fraction was further demonstrated by ninhydrin-Van Slyke analysis and by the use of glutamic acid decarboxylase (15).

An aliquot of the dicarboxylic amino acid fraction was treated with ninhydrin by the usual manometric technique (16), and the liberated CO_2 fixed as barium carbonate by delivery into barium hydroxide. This CO_2 was derived from the α- and β-carboxyl groups of aspartic acid and the α-carboxyl group of glutamic acid. In some experiments, glutamic acid decarboxylase was employed to release only the carbon dioxide of the α-carboxyl group of glutamic acid. This gas was also fixed as above.

Prior to precipitation of the dicarboxylic amino acids, the hydrolysates were treated with ninhydrin and the liberated CO_2 collected and determined. On some of the hydrolysates, a measured aliquot was burned in a combustion furnace packed with copper oxide and the CO_2 collected for the determination of the specific activity of the average amino acid carbons.

All barium carbonate samples were centrifuged, washed, and transferred to steel counting cups as described elsewhere (17). Duplicate samples of carbonate were prepared, transferred, and counted with about 5 per cent accuracy. All radioactivity measurements were made on the solid, dried precipitate, with a thin window Geiger-Müller counter. Counts were corrected for self-absorption and background. On samples of the inorganic carbon from the gas phase and on occasional samples yielding CO_2 in excess of the range of the Van Slyke manometer, the CO_2 was determined gravimetrically by weighing the $BaCO_3$.

Results

All of the experiments to be reported subsequently were carried out in the presence of pyruvate and alanine, and before we were aware of their lack of effect on $C^{14}O_2$ incorporation. The reason for adding pyruvate and alanine originally was to provide optimum conditions (as we thought) for the formation of oxalacetate by the carboxylation of pyruvate and the transamination of oxalacetate to aspartic acid by having an excess of alanine present. However, subsequent experiments in which $C^{14}O_2$ and acetate carboxyl carbon incorporation in the rabbit liver proteins were studied in the presence and absence of added pyruvate and alanine indicated that the incorporation into the liver proteins *in vitro* was not measurably influenced by the presence of an excess of these substrates. Presumably, substrates were present in sufficient quantity in the livers of fasted animals to provide for the formation of an isotopic precursor of the dicarboxylic amino acids.

CO_2 and Acetate Carboxyl-Carbon Incorporation during Incubation—The incorporation of CO_2 and acetate carboxyl carbon into liver proteins *in vitro* was found to be considerable at the end of 1 hour of incubation and increased for at least 4 hours.

In Table I are presented data from experiments in which the incorporation of CO_2 and acetate carboxyl carbon radioactivity are compared. In Column 3 is given the total radioactivity added to each flask as $Na_2C^{14}O_3$ (experiments (a)) or carboxyl-labeled acetate (experiments (b)). Column 4 shows the specific activity of the CO_2 in the flasks at the end of the incubation period. In the case of the "acetate" flasks, this radioactivity represents the CO_2 resulting from oxidation of acetate and amounts to 9 to 19 per cent of the total added counts. The raw data in Columns 5 and 6 are the specific activities of the CO_2 obtained by treating the hydrolysates and the dicarboxylic amino acid fractions, respectively, with ninhydrin. For comparison of the degrees of incorporation of CO_2 and acetate, the data in Column 6 have been corrected for those counts due to incorporation of $C^{14}O_2$ resulting from acetate combustion. In calculating this correction, two assumptions are made. First, the rate of CO_2 incorporation is assumed to be the same in the several vessels in each experiment. Second, it is assumed that the final specific activity of CO_2 in the acetate flasks was that present throughout the incubation. Since these flasks contain no radioactive CO_2 at the beginning of the experiments, the calculated corrections are somewhat high. The calculated incorporations of radioactivity derived from acetate into liver slice proteins, therefore, are conservative estimates and may be considerably greater than shown here.

TABLE I

In Vitro Incorporation of $C^{14}O_2$ and $CH_3C^{14}OONa$ into Amino Acids of Rabbit Liver Slice Proteins

Pyruvate, 40 mM per liter. The experiments marked (a) had C^{14} added as $Na_2C^{14}O_3$; the experiments marked (b) had C^{14} added as $CH_3C^{14}OONa$. Specific activities expressed as counts per minute per mM of carbon. Each flask contained 0.22 mM total CO_2.

Experiment No.	Incubation time	C^{14} added	Final specific activity of inorganic C	Specific activity of carboxyl CO_2*		Ratio $\frac{(6)}{(5)}$	Carboxyl specific activity		
				Hydrolysate	Glutamic + aspartic acids		Derived from CO_2	Derived from acetate	Per 100,000 counts per minute per vessel
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
	hrs.	*counts per min.*							
1a	2	71,500	325,000		1030				1440
1a	4	71,500	325,000	915	2780	3.0			3890
1b	4	21,800	18,700	140	525	3.7	160	365	1670
2a	2	57,000	258,000		560				980
2a	4	57,000	257,000	330	750	2.3			1310
2b	2	40,000	24,800	85	240	2.8	54	186	510
2b	4	40,000	26,000	120	500	4.2	75	425	1120
3a	4	36,000	165,000	194	360	1.9			1000
3b	4	19,000	8,400		84		18	66	350
3b	4	19,000	10,600		83		23	60	320

* CO_2 liberated by ninhydrin; 7 minutes, 100°, pH 2.5.

The corrections in Column 8 were obtained by multiplying the specific activity of the carboxyl carbon dioxide of the dicarboxylic amino acids by the ratio of specific activities of the inorganic carbon dioxide in the corresponding "acetate" and "CO_2" flasks incubated for the same period of time. In Column 9 this correction has been subtracted from the corresponding value in Column 6. For direct comparison, the incorporated radioactivity from CO_2 and acetate are expressed in Column 10 on the basis of 100,000 total counts per minute added to each vessel.

The data in Column 10 indicate that there has been incorporation of acetate carboxyl carbon into the carboxyl group of glutamic and aspartic acids by a mechanism not involving CO_2 and that this occurs at a rate about one-half to one-third that of CO_2 incorporation.

These data yield another fact of particular interest. In Column 7, the ratios of the specific activity of the dicarboxylic amino acid fraction to that of the total hydrolysate have been calculated and average 3.0. Block (18) has reported that the glutamic acid and aspartic acid contents of liver are 12.2 per cent and 6.9 per cent, respectively. If all the radioactivity found in the carbon dioxide liberated by ninhydrin oxidation of the total hydrolysate resided in the α-carboxyl group of glutamic acid and either or both of the carboxyl groups of aspartic acid, this ratio (Column 7) should be 3.9 on the basis of the figures reported by Block. It appears, therefore, that a large part of the incorporated radioactivity may be accounted for in the dicarboxylic amino acids, since the ratio in Column 7 should be unity instead of 3.0 if all the amino acids were equally radioactive in their carboxyl positions. As will be shown below, the specific activities of the α-carboxyl group of glutamic acid and of both of the carboxyl groups of aspartic acid taken together are approximately the same.

Distribution of C^{14} between Glutamic and Aspartic Acids—A number of large scale experiments with $Na_2C^{14}O_3$ were performed with rabbit liver slices in order to prepare sufficient hydrolysate for more detailed study of the dicarboxylic amino acid fraction. In these experiments, about 2 to 3 gm. of slices were employed per vessel and the other components of the system were increased proportionately. Barium fractions were prepared as described above. On these fractions were determined the total amino nitrogen (NH_2-N) by the nitrous acid method of Van Slyke (19), the ninhydrin-liberated carbon dioxide (COOH-N), and the carbon dioxide liberated by squash glutamic acid decarboxylase (15). The aspartic acid content was calculated as one-half the difference between the decarboxylase and ninhydrin values.

The analytical results are presented in Table II. The total dicarboxylic amino acid NH_2-N of the barium fraction, Column 5 (calculated from Columns 3 and 4), is in essential agreement with the values found by direct nitrous acid analysis, Column 6. From the agreement of these values, it is apparent that the barium fractions contained no significant amounts of α-NH_2-N other than those of glutamic and aspartic acids. The absence of other amino acids in the fraction was further demonstrated by filter paper chromatography, as referred to above.

The radioactivity data are also presented in Table II. These show that the specific activity of the glutamic acid α-carboxyl carbon and the average specific activity of the ninhydrin-liberated CO_2 are in fair agreement. From this, it may be inferred that the average specific activity of the two aspartic acid carboxyl carbons is approximately equal to that of the glutamic acid α-carboxyl carbon. No evidence is available on the radioactivity of the γ-carboxyl carbon of glutamic acid.

TABLE II

Composition of Dicarboxylic Amino Acid Fraction and Distribution of C^{14} between Glutamic and Aspartic Acids

Rabbit liver slices incubated 4 hours in medium containing 40 mM of pyruvate per liter and 7.5 mM of alanine per liter.

Experiment No. (1)	Analytical data*					Radioactivity data	
	Ninhydrin N (2)	Glutamic decarboxylase COOH-N (3)	Aspartic acid α-COOH-N $\frac{(2)-(3)}{2}$ (4)	Calculated dicarboxylic amino acid NH_2-N (3) + (4) (5)	Found NH_2-N (6)	Ninhydrin CO_2 (7)	Glutamic decarboxylase CO_2 (8)
						counts per min. per mM	*counts per min. per mM*
1	21.4	7.8	6.8	14.6	15.4	1140	940
2	43.1	16.4	13.4	29.8	30.4	1000	810
3	34.1	11.6	11.3	22.9	23.4	2030	2170

* For direct comparison of the results of the three analytical procedures, the data are expressed as micromoles of nitrogen. All analyses were performed on aliquots of the same size.

As was mentioned under "Methods," aliquots from some of the hydrolysates were combusted for the determination of the average specific activity of all the carbon atoms in the hydrolysate. The results of these experiments are given in Table III. The average carbon chain length of the amino acids present is approximately 4.6 as calculated from the mM of carboxyl carbon (Column 3) and the mM of carbon dioxide derived by combustion (Column 4). When the specific activity of the carbon dioxide resulting from the combustion is multiplied by 4.6 (Column 8) to make the results comparable to the ninhydrin-liberated carbon dioxide values, the resulting specific activities are from 2 to 3 times those of the corresponding carboxyl carbon samples (compare Columns 8 and 6).

It has been demonstrated (Table I) that a large part of the radioactivity was incorporated into the carboxyl groups from carbon dioxide and acetate residues in the α-carboxyl group of glutamic acid and in one or both of the carboxyl groups of aspartic acid. The results in Table III suggest that additional carbon atoms in the average protein molecule of the rabbit liver slices have been in equilibrium with environmental carbon dioxide or acetate, since the radioactivity calculated as the counts per minute per average carbon chain (Column 8) exceeds the specific activity of the carboxyl carbon dioxide (Column 6).

It should be emphasized that the hydrolysate employed, although prepared from protein which has been thoroughly washed and defatted, may contain trace amounts of non-protein components containing radioactivity. Thus, for example, both CO_2 and acetate have been shown to be precursors of the purine ring structure (20), and radioactivity from these sources may possibly be present in the non-protein moiety of the nucleoproteins. In the case of ninhydrin-liberated carbon dioxide, this would not affect the results, since this reagent is highly specific for the amino acid molecule. However, it would apply to the combusted samples and the interpretation of the results presented here must, therefore, await further study through the isolation of individual pure amino acids.

TABLE III

Comparison of Specific Activities of CO_2 Samples Obtained by Ninhydrin Treatment and Combustion of 0.1 Ml. Aliquots of Hydrolysates from Rabbit Liver Slice Proteins

Pyruvate, 40 mM per liter. The experiments marked (a) had C^{14} added as $Na_2C^{14}O_3$; the experiments marked (b) had C^{14} added as $CH_3C^{14}OONa$.

Experiment No. (1)	Incubation time (2)	Ninhydrin CO_2 (3)	Combustion CO_2 (4)	Carbon chain length (5)	Ninhydrin CO_2 (6)	Combustion CO_2 (7)	4.6 × (7) (8)
	hrs.	*mM*	*mM*		*counts per min. per mM*	*counts per min. per mM*	
1a	4		0.25		920	360	1650
1b	4	0.050	0.24	4.8	140	100	460
2a	4	0.059	0.24	4.1	330	175	810
2b	2	0.072	0.34	4.7	80	37	170
2b	4	0.072	0.33	4.6	120	92	420

In an attempt to localize at least a portion of this "extra" radioactivity, the specific activity of the guanidine carbon of arginine has been determined in experiments with added $Na_2C^{14}O_3$. This carbon was released by treatment of the hydrolysate with purified arginase (21) followed by release of the urea carbon with urease in the manometric Van Slyke apparatus (22). The results reported in Table IV indicate that the guanidine carbon has almost twice the specific activity of the glutamic and aspartic carboxyl carbons. This would account for a considerable proportion of the radioactivity found in the protein hydrolysates not attributable to carboxyl carbons.

TABLE IV

Comparison of in Vitro Incorporation of Radioactive CO_2 in Guanidine Carbon of Arginine and Carboxyl Groups of Glutamic and Aspartic Acids

10 ml. of medium, 40 mM of pyruvate per liter. The radioactivity added as $Na_2C^{14}O_3$. Incubation time, 4 hours.

Experiment No.	Specific activity of final inorganic CO_2	Ninhydrin CO_2* of dicarboxylic amino acids	Guanidine C of arginine	Guanidine C specific activity / Carboxyl C specific activity
	counts per min. per mM	*counts per min. per mM*	*counts per min. per mM*	
1	294,000	2030	3700	1.8
	360,000	2640	4700	1.8
2	360,000	1220	2700	2.2
	350,000	970	1300	1.4

* α-Carboxyl carbon of glutamic acid plus both carboxyl carbons of aspartic acid.

TABLE V

Effect of Anaerobiosis and Mincing on Incorporation of $C^{14}O_2$ in Rabbit Liver Protein Dicarboxylic Amino Acids

Incubated for 4 hours in medium containing 40 mM of pyruvate per liter and 7.5 mM of alanine per liter.

Experiment No.	Specific activity of CO_2	Conditions	Specific activity of ninhydrin CO_2 of dicarboxylic amino acids
	counts per min. per mM		*counts per min. per mM*
1	350,000	Control	1230
		5% CO_2-95% N_2	0
		Mince	360
2	450,000	Control	2780
		5% CO_2-95% N_2	0
3	340,000	Control	1310
		5% CO_2-95% N_2	0
		Mince	750

Effect of Anaerobiosis and Mincing—In Table V are presented data from experiments on the effect of anaerobiosis and of mincing the tissue slices on the incorporation of $C^{14}O_2$ in the carboxyl groups of the dicarboxylic amino acids. The liver was minced at room temperature with the edge of a spatula on a porcelain plate until reduced to a semifluid sludge. An amount of liver corresponding to the number of slices used

TABLE VI

Effect of Addition to Medium of Non-Isotopic Glutamic and Aspartic Acids and of Glutamine and Asparagine on Incorporation of $C^{14}O_2$ in Rabbit Liver Protein Dicarboxylic Amino Acids

Pyruvate, 40 mM per liter; alanine, 7.5 mM per liter. The specific activity of CO_2 in Experiment 1 is 402,000 counts per minute per mM; in Experiment 2, 325,000 counts.

Experiment No.	Incubation time	Additions (as sodium salts), pH 7.4	Specific activity of ninhydrin-liberated CO_2 of dicarboxylic amino acids
	hrs.		*counts per min. per mM*
1	2	None (control)	1450
	5	" "	3560
	5	2 mg. each aspartic and glutamic acids	3580
	5	5 " " " " " "	3100
	5	10 " " " " " "	3640
2	2	None (control)	1030
	4	" "	2780
	4	8 mg. each glutamine and asparagine	2200
	4	8 " " glutamic and aspartic acids	2210

TABLE VII

Effect of Addition of Non-Isotopic Malate and Oxalacetate to Rabbit Liver Slice System

Pyruvate, 40 mM per liter; alanine, 7.5 mM per liter.

A. Malate; Specific Activity of CO_2 = 243,000 Counts per Minute per mM

Incubation time	Added Na malate (pH 7.4)	Specific activity of ninhydrin-liberated CO_2 of dicarboxylic amino acids
hrs.	*mM per l.*	*counts per min. per mM*
2	None	1030
4	"	1750
4	5	1900
4	12	1300
4	25	2000

B. Oxalacetate; Incubated for 4 Hours

Experiment No.	Added Na oxalacetate (pH 7.4)	Specific activity of CO_2	Specific activity of ninhydrin-liberated CO_2 of dicarboxylic amino acids
	mM per l.	*counts per min. per mM*	*counts per min. per mM*
1	None	165,000	370
	5		330
	25		210
2	None	225,000	1250
	25		590
3	None	140,000	1060
	25		420
	50		450

in the control flask was then transferred to 3 ml. of medium. In the anaerobic vessels, flushed with 5 per cent CO_2-95 per cent nitrogen, no radioactivity was found in the carboxyl CO_2 of the barium fraction. The over-all process, therefore, is dependent on the presence of oxygen confirming the results of others (3, 5). Mincing the tissue caused a decrease in incorporation to about one-half to one-third that obtained in the control vessels.

Effect of Various Substrates—In an attempt to determine possible intermediates and precursors of the radioactive dicarboxylic amino acids found in the slices, we have added (in non-isotopic form) glutamic and aspartic acids, asparagine and glutamine, malate, and oxalacetate.

As the data in Table VI show, the addition of as much as 10 mg. (about 23 mM per liter) each of glutamic and aspartic acids and 8 mg. (about 20 mM per liter) of glutamine and asparagine to the liver slice system caused no decrease in the specific activity of the dicarboxylic amino acid carboxyl carbons.

The results of the experiments with added malate and oxalacetate are shown in Table VII. No effect on the specific activity of the dicarboxylic amino acid carboxyl carbons was observed with malate, but oxalacetate alone of all the added substrates caused a decrease in specific activity.

DISCUSSION

Evans and Slotin (23) working with C^{11} showed that, when pigeon liver mince was incubated with radioactive CO_2, some of the radioactivity was found in the carboxyl groups of the free amino acids and was released by the action of ninhydrin or chloramine T. Although these experiments provided early evidence that the enzymatic incorporation of CO_2 into amino acids was possible *in vitro*, no examination of the protein was made at that time.

Experiments *in vivo* by Delluva and Wilson (24) have demonstrated the rapid appearance of isotopic carbon atoms from administered CO_2 in the dicarboxylic amino acids of tissue proteins. Rittenberg and Bloch (25) have shown that, after feeding carboxyl-labeled acetic acid to rats, aspartic and glutamic acids isolated from the tissues of the animals contained isotope in the carboxyl positions. The results presented above demonstrate the incorporation of isotopic CO_2 and acetate into liver proteins *in vitro*. They also show that, of the incorporated radioactivity found in the carboxyl groups of the liver protein amino acids, the bulk is localized in glutamic and aspartic acids in about equal amounts.

The reaction through which the CO_2 molecules pass in reaching their final positions in the protein molecule are still obscure, due to the failure of added pyruvate, alanine, glutamate, aspartate, glutamine, asparagine, or malate to affect the specific activity of the carboxyl carbons of the protein dicarboxylic amino acids. The lowered specific activity observed in the presence of oxalacetate may indicate an actual isotopic dilution effect on a product of the CO_2-fixing reaction, or may simply indicate a toxic effect of high concentration of this substance on the enzyme systems involved in the assimilation of CO_2 into liver proteins. Such an effect has been reported by Buchanan *et al.* in studies on acetoacetate oxidation (26).

SUMMARY

1. Incubation of rabbit liver slices with $NaHC^{14}O_3$ or $CH_3C^{14}OONa$ results in the incorporation of radioactivity into the carboxyl groups of the protein amino acids. This incorporation continues progressively for at least 4 hours.
2. A major part of this incorporated carbon is found in the dicarboxylic amino acid fraction. The specific activity of the α-carboxyl group of glutamic acid is approximately equal to the average specific activity of the α- and β-carboxyl groups of aspartic acid.
3. In the absence of oxygen, there is no incorporation of $C^{14}O_2$. The degree of incorporation is decreased markedly by mincing the tissue.
4. The addition of the following non-isotopic substrates had no effect on the degree of incorporation of $C^{14}O_2$: glutamic acid, aspartic acid, glutamine, asparagine, malate, pyruvate, and alanine. Added oxalacetate decreased the incorporation to approximately 50 per cent of the control values.
5. After incubation with both isotopic acetate and bicarbonate, protein hydrolysates contained radioactivity in excess of that accounted for by the dicarboxylic amino acid carboxyl carbon radioactivities.
6. In experiments with $NaHC^{14}O_3$, evidence was obtained showing that the guanidine carbon of arginine has undergone exchange with radioactive carbon.

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THE FORMATION OF CITRATE BY EXTRACTS OF RABBIT KIDNEY CORTEX*

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The metabolic interrelationships of the tricarboxylic acid cycle have received wide-spread attention during the past few years. Curiously, one of the least understood steps in this series of reactions is the original condensation reaction, the formation of citrate or of a related compound. Breusch (1–3) has described various tissue minces containing an enzyme citrogenase which catalyzes the formation of citrate from oxalacetate plus β-keto acids. This enzyme was extracted with 0.5 per cent $NaHCO_3$, and was fairly stable. Wieland and Rosenthal (4) also demonstrated the formation of citrate from oxalacetate, acetoacetate, and Ba^{++}, using a mash of rabbit kidney cortex. Martius (5) described citrate formation from oxalacetate and pyruvate by hog heart, rabbit kidney, or liver pulp. Hunter and Leloir (6), using a washed suspension of dog kidney cortex, demonstrated that α-ketoglutarate oxidation stimulates the conversion of oxalacetate and acetoacetate to citrate, but α-ketoglutarate oxidation was not necessary for the formation of citrate when oxalacetate was the only substrate. They also found that the enzyme was not truly extracted by $NaHCO_3$, but was associated with particles which were sedimented by high speed centrifugation. Kalnitsky (7) obtained increased yields of citrate from oxalacetate in the presence of $MgCl_2$ or fluoroacetate, with rabbit kidney cortex homogenates.

The present communication demonstrates that the enzymes necessary for citrate formation from oxalacetate are present almost entirely in the mitochondria fraction of cortex cells of rabbit kidney. Various optimum conditions for citrate formation are described, including the necessity of inorganic phosphate and Mg^{++} or Mn^{++} for the reaction.

Methods

Tissue Preparation—Rabbits were stunned by a blow on the head and were bled and the kidneys removed immediately. The kidney cortex was weighed and homogenized in 2 volumes of NaCl-KCl solution (2 parts of 0.9 per cent NaCl plus 1 part of 1.15 per cent KCl) in a Potter-Elvehjem

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glass homogenizer (8). The homogenate was then centrifuged at 1500 × gravity for 3 to 5 minutes. The packed precipitate was discarded and the cloudy supernatant utilized as the enzyme suspension.

For some experiments, the enzyme suspension was dialyzed in a cellophane bag against distilled water at 0° for 15 or 20 minutes, as indicated. Washed preparations of the enzyme suspension were obtained by centrifuging at 19,000 to 20,000 × gravity for 5 to 10 minutes. The resulting supernatant was discarded and the smooth, creamy precipitate was made up to two-thirds the volume of the original supernatant with NaCl-KCl solution.

The activity of all these enzyme preparations was determined by incubating, in 50 ml. Erlenmeyer flasks. These were shaken at 120 3.5 cm. strokes per minute at approximately 30°. The time of each experiment was 1 hour, unless otherwise indicated.

Fractionation of Cellular Components—The original homogenate of kidney cortex was fractionated essentially as described by Claude (9, 10), Hogeboom *et al.* (11), and Schneider (12). The procedure used in isolating the mitochondria and microsome fractions was as follows: The homogenate was centrifuged for 3 minutes at 1500 × gravity to sediment the nuclei, whole cells, and débris. This sedimented fraction was then washed three times with 15 ml. of alkaline water (12) and centrifuged 3 minutes at 1500 × gravity, the supernatant fluid being discarded. This washed sediment, plus 3 times its own volume of NaCl-KCl solution, was termed the residue. The original supernatant was centrifuged at 1500 × gravity for 3 minutes. This process was carried out three times, the sediment being discarded each time. The supernatant (containing mitochondria and microsomes) was then centrifuged at 2400 × gravity for 15 to 20 minutes to sediment the mitochondria or large granules. The mitochondria precipitate was washed with 15 ml. of NaCl-KCl solution and centrifuged as before. This precipitate, plus 3 times its own volume of NaCl-KCl solution, was called the mitochondria fraction. The supernatant from the mitochondria fraction was then centrifuged at 19,000 to 20,000 × gravity for 5 minutes. The small precipitate (mitochondria) was discarded, and the supernatant was centrifuged at 19,000 to 20,000 × gravity for 90 minutes. The jelly-like precipitate obtained, made up with an approximately equal volume of NaCl-KCl solution, was termed the microsome fraction. All centrifugations were carried out in a cold room at 0°.

The mitochondria were examined under the microscope, with and without stain. The Janus green stain for mitochondria (13) was positive, and there was practically no other material present.

Chemical Methods—Citrate was determined as previously described (7). Oxalacetate was prepared according to the method described by Krampitz and Werkman (14) and was dissolved and neutralized immediately before use. Acetoacetate was prepared according to Ljungren (15), and was also dissolved and neutralized before use. Lithium pyruvate was prepared according to Wendel (16).

Results

Effect of Various Substrates

Various substrates have been employed in attempts to increase citrate formation from oxalacetate with animal tissues (1, 4–6). Pyruvate and acetoacetate increased citrate formation, but acetate did not. Under the conditions of our experiments, acetate, as well as pyruvate and acetoacetate, increased citrate formation from oxalacetate (Table I). Somewhat similar increases with acetate have also been obtained by Lehninger,[1] using rat liver tissue. Caproate was slightly inhibitory, possibly due to its surface action. However, the largest yields of citrate from oxalacetate were obtained by the addition of fluoroacetate (Table I).

TABLE I

Citrate Formation from Various Substrates

Experiment No.	Oxalacetate added	Other substrates added		Citrate formed	
				No fluoroacetate	Plus fluoroacetate
	μM		*μM*	*μM*	*μM*
1	100			7.1	26.0
	100	Pyruvate	100	14.1	28.4
	100	Acetoacetate	50	11.6	26.6
2	100			12.5	30.6
	100	Acetate	100	17.5	
	100	"	300	18.5	26.5
	100	"	500	18.0	
3	30			0.4	
		Acetate	300	0.4	
	30	"	300	6.5	
	50			4.3	22.4
	50	Acetate	300	9.3	

Tissue suspension 2.0 ml. (Experiment 1), 1.5 ml. (Experiment 2), 2.0 ml. of washed suspension (Experiment 3); substrates in indicated amounts; fluoroacetate 100 μM (0.02 M); phosphate buffer, pH 7.6, 0.03 M; $MgSO_4$, 0.006 M (Experiment 2), 0.01 M (Experiment 3); total volume 5.0 ml.; atmosphere, air.

Optimum Substrate and Enzyme Concentrations

Despite the instability of oxalacetate the enzyme system is easily saturated, since the optimum concentration of oxalacetate was 0.02 to 0.025 M

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[1] Lehninger, A., private communication.

(Fig. 1). The activity of the enzyme was proportional to the enzyme concentration, up to 1.5 ml. of the enzyme preparation (Fig. 2).

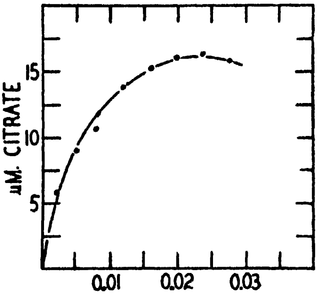

FIG. 1

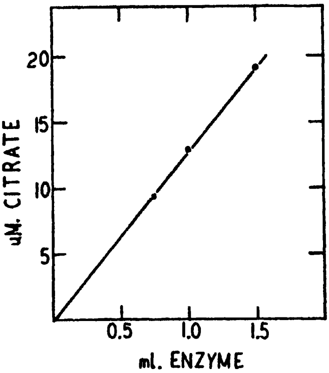

FIG. 2

FIG. 1. Effect of varying concentration of substrate on citrate formation. Enzyme preparation 1.6 ml., oxalacetate in indicated concentrations, fluoroacetate 0.01 M, phosphate buffer 0.016 M, pH 7.50; total volume, 4.6 ml.

FIG. 2. Effect of varying amounts of enzyme on citrate formation. Oxalacetate, 0.02 M; same conditions as in Fig. 1.

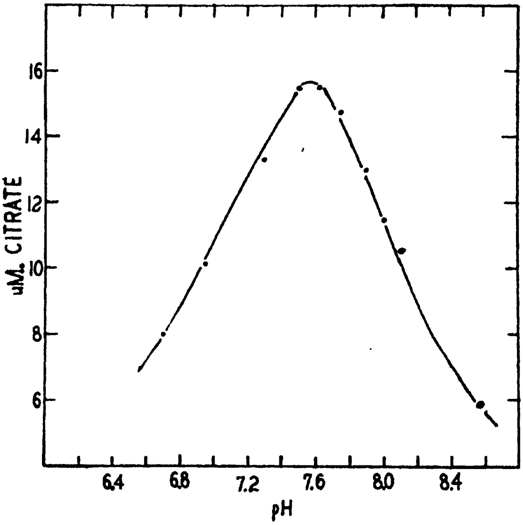

FIG. 3. The effect of varying pH on citrate formation. Enzyme preparation 2.0 ml., oxalacetate 0.02 M, fluoroacetate 0.02 M, phosphate buffers 0.04 M, pH determined at the end of the experimental period, time 55 minutes; total volume, 5.0 ml.; atmosphere, air.

Optimum pH for Citrate Formation from Oxalacetate

The effects of varying pH on citrate formation from oxalacetate are presented in Fig. 3. The pH optimum is definitely in the alkaline range, between pH 7.2 and 7.9, with the peak at pH 7.5 to 7.6. The pH values were determined at the end of the experimental period. The results are in agreement with the optimum pH values obtained by Breusch and Keskin (3) for citrate formation from oxalacetate plus acetoacetate, by the enzyme citrogenase, in pigeon muscle mash.

Influence of Oxygen

The effect of various gaseous atmospheres on citrate formation are presented in Table II. Various investigators (4, 17) have reported that oxygen was necessary for citrate formation from oxalacetate plus acetoacetate, whereas others (3, 6) have shown that the same reaction can take place either aerobically or anaerobically. Hunter and Leloir (6) have indicated that, in the presence of excess oxalacetate (100 μM) acting as an oxidant, citrate formation can also take place anaerobically. In our experiments, in the presence of 100 μM of oxalacetate and with a less concentrated kidney cortex suspension than employed by Hunter and Leloir, 52 to 55 per cent less citrate was formed in an atmosphere of nitrogen than in oxygen or air (Table II).

TABLE II

Effect of Various Gaseous Atmospheres on Citrate Formation

Gaseous atmosphere	Citrate formed
	μM
Air	18.5
O_2	17.5
N_2	8.4

Tissue suspension 1.6 ml. per vessel; oxalacetate 0.02 M (100 μM); fluoroacetate 0.01 M, phosphate buffer, pH 7.50, 0.015 M; total volume 4.6 ml.

The enzyme preparation is fairly stable, and retains all its activity after standing 1 hour at room temperature (approximately 28–30°) or 2 hours at 0–2°.

Effect of Inorganic Phosphate and Various Buffers on Citrate Formation

In early experiments on the effects of various buffers on citrate formation, it was noted that phosphate buffer was most effective. With undialyzed extracts, inorganic phosphate increased citrate formation somewhat (Table III). After dialysis of the extract for 15 or 20 minutes, citrate formation from oxalacetate was markedly increased on the addition of inorganic phosphate. This effect of phosphate was not due to maintenance of the pH of the system, since the pH values determined at the end of the experimental period were not markedly different in the presence or absence of phosphate, The pH in these experiments was maintained with bicarbonate-CO_2 buffer. Veronal and borate buffers, at 0.01 M concentrations (with inorganic phosphate added), were somewhat inhibitory (Table III). At 0.04 M concentrations, these buffers were definitely inhibitory, the inhibitions obtained ranging from 40 to 65 per cent. Collidine buffer (2,4,6-trimethylpyridine) has been reported to be non-toxic for pyruvate oxidation by liver slices (18). The sample we used was toxic, 0.01 M concentration producing 60 per cent inhibition of citrate formation.

TABLE III

Effect of Inorganic Phosphate on Citrate Formation by Dialyzed Suspension of Rabbit Kidney Cortex

Experiment No.	Conditions	Buffer added	pH	Citrate formed	Per cent of total
				μM	
1	Undialyzed		7.84	10.5	
		Phosphate	7.80	14.8	
	Dialyzed, 15 min.		8.10	4.0	
		Phosphate	7.95	12.6	
2	Dialyzed, 20 min.		7.28	5.7	34
		Phosphate	7.21	16.8	100
		Veronal	7.25	3.4	20
		Borate	7.35	4.3	26
		Veronal + phosphate	7.25	12.5	74
		" + " (0.006 M)	7.25	6.1	36
		Borate + "	7.21	9.1	54

Each vessel contained 2 ml. of tissue suspension; oxalacetate and fluoroacetate 0.02 M, bicarbonate-saline solution 1.5 ml. in Experiment 1, 1.0 ml. in Experiment 2; all buffers, 0.01 M, except as indicated; atmosphere, 5 per cent CO_2 in O_2; total volume 5.0 ml.; pH determined after 55 minutes; $CCl_3 \cdot COOH$ then added and citrate determined.

Necessity of Mg^{++} or Mn^{++} for Citrate Formation

The effects of similar dialysis periods upon the activity of different preparations varied widely. This variation was probably due to the different degrees of subdivision of the particles in the tissue suspensions, as a result of the wearing down of the homogenizers used. Some preparations were largely inactivated after dialysis for 25 minutes, whereas other preparations were only slightly inactivated after 35 minutes dialysis but were completely inactivated after dialysis for 55 to 60 minutes. More consistent results were obtained by washing the preparation, instead of dialyzing it. With washed preparations, the necessity of Mg^{++} or Mn^{++} for citrate formation could readily be demonstrated (Table IV).

Optimum Concentrations of Mg^{++}, Mn^{++}, and Inorganic Phosphate

The effects of various concentrations of Mg^{++} and Mn^{++} on citrate formation are presented in Table V. The effectiveness of Mg^{++} and Mn^{++} additions showed an increase up to a concentration of 6 to 8 × 10^{-3} M, beyond which further additions had no influence. Potter *et al.* (19) found that 3.3 × 10^{-3} M $MgCl_2$ was the optimum concentration for oxalacetate oxidation. Approximately this same concentration, 6 × 10^{-3} M inorganic phosphate, was also optimum for citrate formation (Table VI).

TABLE IV

Effect of Mg^{++} and Mn^{++} on Citrate Formation

Conditions	Citrate formed
	μM
Oxalacetate	3.1
" + Mg	30.9
" + Mn	20.0

Washed tissue preparation 1.5 ml.; oxalacetate and fluoroacetate 0.02 M, phosphate buffer, pH 7.6, 0.03 M; $MnSO_4$ or $MgSO_4$, 0.006 M; total volume 5 ml.; atmosphere, air.

TABLE V

Effect of Various Concentrations of Mn^{++} and Mg^{++} on Citrate Formation

Concentration of Mg^{++} or Mn^{++}	Citrate formed	
	Plus Mn^{++}	Plus Mg^{++}
M	μM	μM
	1.5	4.8
1 × 10^{-4}	1.3	6.0
5 × 10^{-4}	5.3	
1 × 10^{-3}		6.4
3 × 10^{-3}	6.8	12.8
6 × 10^{-3}	24.5	29.7
8 × 10^{-3}	23.8	33.7
1 × 10^{-2}	24.0	33.7

Washed kidney suspension 1.5 ml.; fluoroacetate 0.02 M, phosphate buffer, pH 7.6, 0.03 M; oxalacetate 0.02 M, Mg^{++} or Mn^{++} in indicated concentrations; total volume 5.0 ml.; temperature 32°; atmosphere, air.

Formation of Citrate by Various Fractions of Rabbit Kidney Cortex Cell

The enzyme suspensions employed in this paper roughly resembled the mitochondria and microsome fractions described by Claude (9, 10), Hogeboom *et al.* (11), and Schneider (12), while the washed tissue suspension corresponded with the mitochondria fraction described by these authors. Accordingly, purified preparations of a residual fraction (containing nuclei, whole cells, and débris), a mitochondrial fraction, and a microsome fraction were obtained by fractionating homogenates essentially as described by these workers (see "Methods"). Each fraction was then tested for citrate formation. The results are presented in Table VII. Small amounts of citrate were formed by the residue fraction, very large amounts of citrate by the mitochondria fraction, and little or no citrate was formed by the microsome fraction. It is evident from these data that the enzymes responsible for citrate formation from oxalacetate are almost entirely located in the mitochondria fraction of the cells of rabbit kidney cortex.

TABLE VI

Effect of Various Concentrations of Phosphate on Citrate Formation

Phosphate	Citrate formed
M	μM
5×10^{-4}	2.6
1×10^{-3}	1.8
3×10^{-3}	5.1
6×10^{-3}	23.7
8×10^{-3}	23.6
1×10^{-2}	23.9
1.5×10^{-2}	26.9

Washed kidney suspension 1.5 ml., fluoroacetate 0.02 M, phosphate in indicated concentrations, Mn^{++} 0.006 M; $NaHCO_3$ 0.018 M, oxalacetate 0.02 M, total volume 5.0 ml.; temperature 32°; atmosphere 95 per cent O_2, 5 per cent CO_2.

TABLE VII

Citrate Formation by Various Fractions of Homogenates of Rabbit Kidney Cortex

Tissue fraction	Suspension	Citrate formed
	ml.	μM
Residue (nuclei, débris, unbroken cells)	2.0	2.0
" " " " "	2.0	2.2
" " " " "	3.0	2.2
" " " " "	4.0	2.7
Mitochondria	1.8	39.3
"	2.0	45.0
Microsomes	2.0	0.9

Tissue suspensions of the various fractions in indicated amounts; oxalacetate 0.02 M, fluoroacetate 0.02 M, phosphate buffer, pH 7.2, 0.03 M; $MgCl_2$ 0.01 M; total volume 5.0 ml.; temperature 30.1°; atmosphere, air.

The yield of 45 μM of citrate from 100 μM of oxalacetate by the mitochondria preparation represents a 90 per cent conversion of oxalacetate to citrate.

DISCUSSION

Citrate formation from oxalacetate is commonly accepted to occur via a combination of reactions (1) and (2) or reactions (1), (3), and (4):

$$\text{Oxalacetate} \rightarrow \text{pyruvate} + CO_2 \tag{1}$$

$$\text{Pyruvate} + \text{oxalacetate} \rightarrow \text{procitrate} \rightarrow \text{citrate} + CO_2 \tag{2}$$

$$\text{Pyruvate} \xrightarrow{[O]} \text{2-carbon compound} + CO_2 \tag{3}$$

$$\text{2-Carbon compound} + \text{oxalacetate} \rightarrow \text{procitrate} \rightarrow \text{citrate} \tag{4}$$

Actually, there is no evidence for the occurrence of a 7-carbon precursor of citrate (reaction (2)). One possible 7-carbon intermediate, α-keto,4-hydroxy,4-carboxyadipic acid, has been ruled out by Martius (5). On the other hand, there is evidence for the formation of a 2-carbon unit during the oxidation of pyruvate and of fatty acids, and of the participation of this 2-carbon compound in the formation of citrate (20–23).

If citrate were formed from oxalacetate by a union of oxalacetate with a 2-carbon residue (reaction (4)), then the function of oxygen in citrate formation would be clearer. Oxygen would then be needed for the oxidative decarboxylation of pyruvate (reaction (3)).

The question remains, however, as to the function of inorganic phosphate in the formation of citrate. Inorganic phosphate is not necessary for the decarboxylation of oxalacetate to pyruvate (reaction (1)) (14, 24–26). Several investigators (27–31) have demonstrated that inorganic phosphate is necessary for pyruvate oxidation. However, it has not been determined whether the inorganic phosphate is necessary in the oxidative conversion of pyruvate to the hypothetical 2-carbon intermediate, in the actual condensation reaction, or in a reaction further on down the reaction chain, for example, the oxidation of α-ketoglutarate to succinate (32). Stumpf *et al.* (33, 34) have described preparations obtained from pigeon breast muscle and bacteria which oxidize pyruvate to acetate. No inorganic phosphate is needed for that reaction. This enzyme system is apparently not present in preparations of rabbit kidney cortex which oxidize pyruvate by way of the citric acid cycle (35).[2] However, inorganic phosphate is necessary for acetate oxidation by a similar preparation of rabbit kidney cortex.[3] It will be difficult to determine whether inorganic phosphate is necessary for the oxidative conversion of pyruvate to an intermediate 2-carbon compound, or for the condensation reaction, until the actual intermediate is isolated and the substrates for the condensation reaction determined. In any case, the necessity of inorganic phosphate for citrate formation implies a phosphorylated precursor of citrate. This would probably be an unsymmetrical compound. Other investigators (36, 37) have previously suggested the occurrence of unsymmetrical precursors of citrate to account for the distribution of isotopes in the component members of the citric acid cycle during the oxidation of $CD_3 \cdot COOH$ by yeast (38), and of pyruvate + $C^{13}O_2$ by pigeon liver (36, 37).

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[2] Kalnitsky, G., unpublished results.

[3] Kalnitsky, G., and Elliott, W. B., to be published.

SUMMARY

1. Pyruvate, acetoacetate, and acetate increased citrate formation from oxalacetate by extracts of homogenates of rabbit kidney cortex, but the largest yields of citrate were obtained in the presence of oxalacetate plus fluoroacetate.

2. Optimum concentrations of substrate and tissue were determined. The pH optimum is at 7.5 to 7.7. Air or oxygen is necessary for optimum formation of citrate from oxalacetate.

3. Inorganic phosphate and Mg^{++} or Mn^{++} are necessary for citrate formation from oxalacetate. The optimum concentrations of all these ions were 6×10^{-3} M.

4. Homogenates of rabbit kidney cortex were separated by centrifugation into a residual fraction (containing nuclei, débris, plus unbroken cells), a mitochondria fraction, and a microsome fraction.

5. The enzyme system for the formation of citrate from oxalacetate is found almost entirely in the mitochondria fraction of the kidney tissue.

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DEGRADATION PRODUCTS OF STREPTAMINE: α,γ-DIAMINO-β-HYDROXYGLUTARIC ACID*

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Streptamine, a degradation product of streptomycin, has been characterized as a *meso*-1,3-diamino-2,4,5,6-tetrahydroxycyclohexane (1–4), and this structure has been confirmed by synthesis (5). In a preliminary note (2) we reported the oxidation of N,N'-dibenzoylstreptamine to α,γ-diamino-β-hydroxyglutaric acid. In this paper are reported the details of these experiments together with additional studies of the structure of the glutaric acid derivative.

N,N'-Dibenzoylstreptamine (I) consumed 2 moles of periodate, yielding a crystalline oxidation product which gave a bis-2,4-dinitrophenylhydrazone. In several runs the oxidation product melted at 130–131°. However, occasional samples melted at 143–145° or 163–165°. Each of these materials gave the same hydrazone. The analytical data on the oxidation product indicated a dibenzamidohydroxyglutaraldehyde plus 1 molecule of water. On acetylation a triacetyl rather than a monoacetyl derivative was obtained. These data are best explained by assigning a hydrated cyclic structure to the oxidation product, which then becomes a 2,4,6-trihydroxy-3,5-dibenzamidotetrahydropyran (II). The cyclic structure is also supported by the fact that II is relatively resistant to catalytic reduction. The only homogeneous product isolated from the reaction mixture appeared to be a dibenzamidopentose (IV), which would be formed by reduction of one of the aldehyde groups. The dialdehyde (II) was further characterized by oxidation with bromine water to an α,γ-dibenzamido-β-hydroxyglutaric acid (III). This acid was obtained in good yield. It melted sharply at 199–200° and gave a methyl ester melting at 196–198°. No evidence was obtained for the presence of an isomeric acid.

Since the glutaric acid (III) was a key compound in allocating the position of the amino groups in streptamine, several attempts were made to

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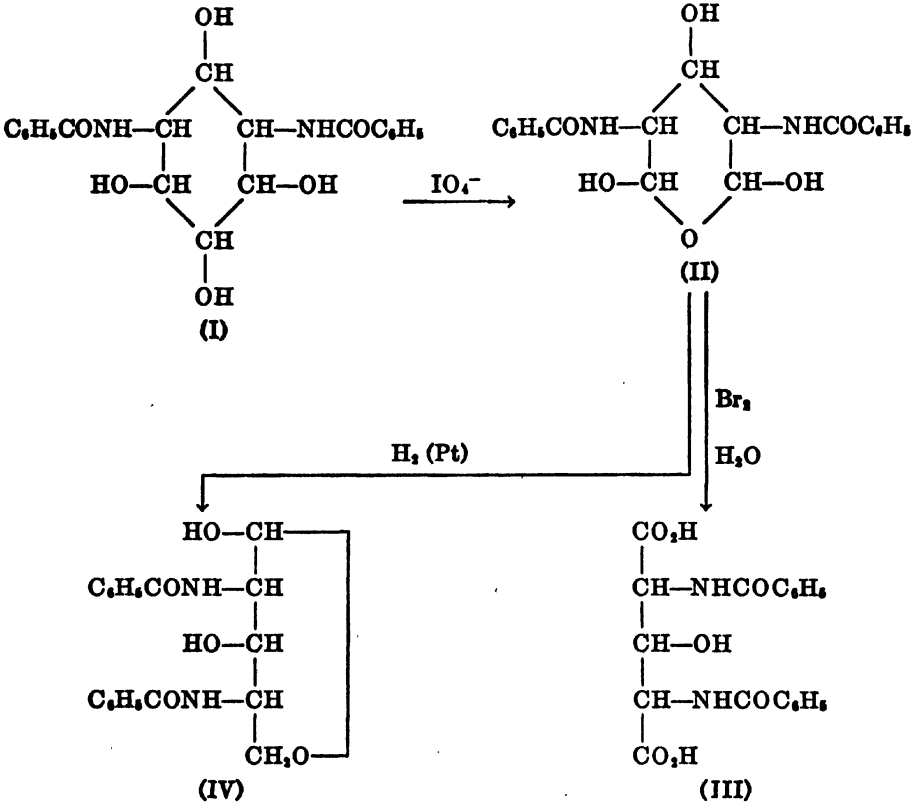
synthesize it. The single stereoisomer which was obtained (6) differed in properties from the degradation product. In view of the fact that α,γ-diamino-β-hydroxyglutaric acid can exist in one racemic and two *meso* forms, further synthetic efforts were postponed. Instead a study was made of the possible conversion of α,γ-dibenzamido-β-hydroxyglutaric acid to α,γ-dibenzamidoglutaric acid (V).



Attempts to dehydrate the dibenzamidohydroxyglutaric acid directly gave unsatisfactory results. However, pyrolysis of dimethyl α,γ-dibenzamido-β-acetoxyglutarate proceeded smoothly with the evolution of acetic acid. The crude, glassy product[1] was reduced catalytically with platinum. The reduced material was not homogeneous, melting over a wide range.

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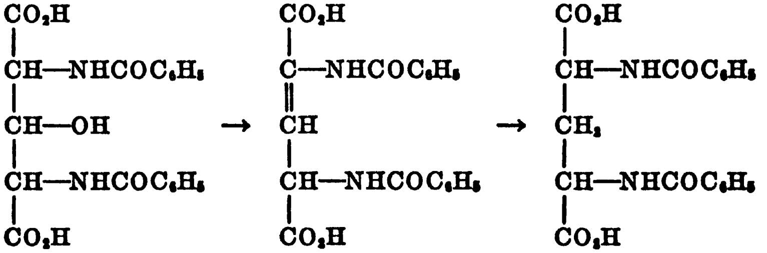
[1] It seemed possible that elimination of acetic acid might yield either an α,β-unsaturated ester or an oxazoline. However, an authentic sample of the oxazoline (m.p. 160–161°) prepared in connection with other work did not undergo reduction with platinum. Furthermore, the infra-red absorption spectrum of the crude pyrolysis product was consistent with the presence of an α,β-unsaturated ester.

By recrystallization from acetone-hexane a fraction was obtained which melted sharply at 178–179°. The mother liquors yielded a mixture melting at 133–155°. From this material individual rosettes could be removed with a spatula. These melted sharply at 150–151°. Both the rosettes and the mixture gave correct analytical data for dimethyl dibenzamidoglutarate. The behavior and properties of the two esters and of the mixture are identical with those of the corresponding synthetic products described recently by Carter, Van Abeele, and Rothrock (7). Thus, the structure of the dibenzamidohydroxyglutaric acid obtained from streptamine has been established by conversion to a known synthetic substance.



$$\begin{array}{ccccc} \mathrm{CO_2H} & & \mathrm{CO_2H} & & \mathrm{CO_2H} \\ | & & | & & | \\ \mathrm{CH{-}NHCOC_6H_5} & & \mathrm{C{-}NHCOC_6H_5} & & \mathrm{CH{-}NHCOC_6H_5} \\ | & & \| & & | \\ \mathrm{CH{-}OH} & \rightarrow & \mathrm{CH} & \rightarrow & \mathrm{CH_2} \\ | & & | & & | \\ \mathrm{CH{-}NHCOC_6H_5} & & \mathrm{CH{-}NHCOC_6H_5} & & \mathrm{CH{-}NHCOC_6H_5} \\ | & & | & & | \\ \mathrm{CO_2H} & & \mathrm{CO_2H} & & \mathrm{CO_2H} \end{array}$$

(III) (V)

Since the degradation of *N*,*N′*-dibenzoylstreptamine involved mild conditions and the α,γ-diamino-β-hydroxyglutaric acid obtained appeared to be homogeneous, it seems unlikely that alteration in stereochemical configuration occurred in the degradation procedure. Therefore, determination of the relationship of the hydroxyl and amino groups in the acid (III) should shed some light on the stereochemistry of the corresponding groups in the streptamine molecule. As a method of attacking this problem, conversion to the corresponding trihydroxyglutaric acid with nitrous acid seemed promising, since several isomers of the latter substance were known as products of the oxidation of pentoses with nitric acid. In preliminary studies α,γ-diamino-β-hydroxyglutaric acid gave theoretical nitrogen values in the Van Slyke amino nitrogen procedure. However, the deamination of α,γ-diamino-β-hydroxyglutaric acid with nitrous acid must have involved further reaction, since no trihydroxyglutaric acid derivative could be obtained from the final product. A study was also made of the deamination of the two α,γ-diaminoglutaric acids. The products were converted to the known dianilides and di-*p*-toluidides of the α,γ-dihydroxyglutaric acids (8). The amino acid obtained from the lower melting dibenzamidoglutaric acid yielded largely *meso*-dihydroxyglutaric acid; that from the higher melting isomer yielded mainly racemic dihydroxyglutaric acid. However, in each case a small amount of the second isomer was produced. Evidently epimerization occurs in the nitrous acid reaction. Since the extent cannot be estimated, this reaction is of little or no value in establishing the configuration of the α,γ-diaminoglutaric acids.

EXPERIMENTAL

Preparation of N,N'-Dibenzoylstreptamine—A solution of 1.7 kilos of low potency streptomycin residues (213 units per mg.) in 4 liters of 10 per cent sulfuric acid was refluxed with mechanical stirring for 3 hours. The black hydrolysate was diluted with 1 volume of acetone and placed in the refrigerator overnight. The brown precipitate which formed was filtered and washed thoroughly with small portions of cold water and 50 per cent methanol. The residue was dissolved in 5 liters of boiling 10 per cent sulfuric acid. The hot solution was decolorized with norit and filtered. The filtrate, neutralized with ammonium hydroxide, was diluted with 1 volume of acetone and placed in the refrigerator overnight. The crystalline product which separated was filtered and washed several times with small portions of cold water and acetone, giving 465 gm. of streptidine sulfate as fine white crystals.

A solution of 400 gm. of streptidine sulfate in 2400 ml. of 6 N lithium hydroxide was refluxed until no more ammonia was evolved (40 hours). Bumping was prevented by mechanical stirring. The hot solution was filtered and the residue (mainly lithium carbonate) was washed thoroughly with hot water. The filtrate was concentrated to 1000 ml. *in vacuo* and adjusted to pH 3.4 with concentrated sulfuric acid. 4 volumes of methanol were added and the solution was stored in the refrigerator for 24 hours. The precipitate which formed was filtered and washed several times with 80 per cent and 100 per cent methanol. Streptamine sulfate was obtained as fine white crystals.

This crude material was converted to polybenzoylstreptamine by the Schotten-Baumann method. For this reaction the crude streptamine sulfate was dissolved in 3 liters of 1 N sodium hydroxide and the solution was treated with 1200 ml. of benzoyl chloride and 4800 ml. of 5 N sodium hydroxide in about 30 equal portions at 5 to 8 minute intervals. The flask was thoroughly shaken and cooled in an ice bath. Ethyl ether was added during the reaction to prevent lump formation by maintaining a thick emulsion of the ingredients. The waxy solid produced was washed repeatedly with water and ether. 760 gm. of amorphous, white polybenzoylstreptamine were recovered.

A solution of this crude product in 10 liters of 0.5 N methanolic sodium hydroxide was refluxed for 4 hours. Insoluble salts were filtered from the hydrolysate, and the filtrate neutralized with concentrated hydrochloric acid to pH 6.9. Salts were again filtered off. The waxy solid obtained by concentrating the filtrate to dryness *in vacuo* was thoroughly triturated with ether and water. The solution of this crude product in 600 ml. of boiling methanol was diluted with 5 volumes of hot water. *N,N'*-Dibenzoylstreptamine slowly separated as fine white rods. The yield was 226 gm. This compound loses its crystalline form at 288–290° and melts with decomposition at 293–295°. The analytical sample was recrystallized from hot water and dried *in vacuo* at 100° for 2 hours.

$C_{20}H_{22}O_6N_2$. Calculated. C 62.17, H 5.74, N 7.25
386.40 Found. " 62.21, " 5.49, " 7.31 (micro-Dumas)
" 0.00 (Van Slyke amino N)

Periodate Oxidation of N,N'-Dibenzoylstreptamine—38.6 gm. (0.1 mole) of *N,N'*-dibenzoylstreptamine were dissolved by mechanical stirring in 5000 ml. of 50 per cent methanol solution containing 64.2 gm. (0.3 mole) of sodium metaperiodate. The mixture was allowed to stand at room temperature until 2.0 ± 0.1 moles of periodate per mole of sample had been consumed (12 to 15 hours). The excess periodate and iodate formed in the reaction were removed by precipitation with lead nitrate. The solution was concentrated to 2500 ml. and upon standing overnight at room temperature 31.0 gm. of 2,4,6-trihydroxy-3,5-dibenzamidotetrahydropyran (II) (83.5 per cent yield) separated as fine white needles. A second crop of 3.5 gm. (9.4 per cent) was obtained by further concentration. The dialdehyde after recrystallization from dry acetone melted at 130–131°. The analytical sample was dried *in vacuo* over phosphorus pentoxide.

$C_{19}H_{20}O_6N_2$. Calculated. C 61.29, H 5.38, N 7.53
372.37 Found. " 61.36, " 5.33, " 7.43

A mixture of 13 mg. of the dialdehyde II and 40 mg. of 2,4-dinitrophenylhydrazine in 10 ml. of absolute ethanol was heated to the boiling point. Concentrated hydrochloric acid (0.2 ml.) was added, and the mixture was warmed in a hot water bath for a few minutes. On cooling, orange needles crystallized from the solution. The crystals were washed free of acid and of the reagent with absolute ethanol. A yield of 20 mg. of the bis-2,4-dinitrophenylhydrazone of II melting at 231–232° was obtained.

$C_{31}H_{26}N_{10}O_{11}$. Calculated. C 52.10, H 3.64, N 19.60
714.60 Found. " 52.16, " 3.96, " 19.79

A mixture of 116 mg. of the dialdehyde II, 2 ml. of pyridine, and 2 ml. of acetic anhydride was allowed to stand at room temperature overnight. The solution was chilled and diluted with water; the resulting yellow precipitate was dissolved in acetone and the solution was decolorized with norit A. The colorless solution was concentrated and water was added to precipitate a white solid. Repeated recrystallization of this material from acetone-water gave needles, melting at 217°.

$C_{25}H_{26}N_2O_9$.	Calculated.	C 60.24, H 5.26, N 5.62
498.48	Found.	" 60.69, " 5.35, " 5.75

Hydrogenation of the dialdehyde II in methanol with Raney's nickel catalyst at room temperature and 30 to 35 pounds pressure for 18 hours reduced only one of the carbonyl groups. The catalyst was filtered off and the alcohol solution was concentrated, giving a crystalline solid. Dilution of the mother liquor with water produced a mixture of products. Repeated recrystallization from hot methanol gave a 20 per cent yield of homogeneous crystalline solid, consisting of shiny plates which melted at 230–231°. This material gave the correct analytical data for 2,4-dihydroxy-3,5-dibenzamidotetrahydropyran.

$C_{19}H_{20}N_2O_5$.	Calculated.	C 64.04, H 5.62, N 7.86
356.37	Found.	" 64.19, " 5.59, " 7.71

Preparation of α,γ-Dibenzamido-β-hydroxyglutaric Acid (III)—A suspension of 16.0 gm. of the dialdehyde II and 30 ml. of bromine in 8000 ml. of water was stirred mechanically until a clear red solution resulted (15 to 20 minutes). Oxidation was allowed to continue at room temperature for 80 hours. Excess bromine was removed by aeration. An excess of calcium carbonate was added and the solution was heated to boiling and filtered. The filtrate was concentrated to about 100 ml. and 9 volumes of ethanol were added, precipitating 17 gm. of the calcium salt of α,γ-dibenzamido-β-hydroxyglutaric acid. This material was suspended in 2 liters of boiling water and 11.5 gm. of oxalic acid dihydrate (100 per cent excess) were added. The precipitated calcium oxalate was removed by filtration, and the filtrate was concentrated to 900 ml. and 2 ml. of concentrated hydrochloric acid were added. The mixture was chilled overnight in the refrigerator, giving 6.0 gm. (36 per cent yield) of α,γ-dibenzamido-β-hydroxyglutaric acid as small, silky white needles melting at 199–200° (micro block). The filtrate was concentrated and cooled in the refrigerator overnight, giving a second crop of 4.4 gm. (26 per cent). The acid was recrystallized twice from hot water. The analytical sample was dried over phosphorus pentoxide *in vacuo* at room temperature for 24 hours and at 78° for 2 hours.

$C_{19}H_{18}O_7N_2$.	Calculated.	C 59.07,	H 4.70,	N 7.25,	neutral equivalent	193
386.35	Found.	" 58.79,	" 4.69,	" 7.03,	" "	192

Preparation of Dimethyl α,γ-Dibenzamido-β-acetoxyglutarate—5.0 gm. of α,γ-dibenzamido-β-hydroxyglutaric acid were converted to the dimethyl ester in the usual manner with use of an ethereal solution of diazomethane.

The white solid obtained after removal of the ether was dissolved in 250 ml. of boiling acetone. The solution was filtered and hot heptane (b.p. 98–110°) was added until the solution was slightly turbid (75 to 100 ml.). After standing at room temperature for several hours a solid mass of white rods crystallized. The first crop weighed 4.6 gm. (86 per cent yield) and melted at 194–196°. A second crop of 0.4 gm. (7 per cent) was obtained by reworking the mother liquor. By recrystallization the melting point of the material was raised to 196–198° (micro block). A mixed melting point (1:1) with starting material was 178–180° (micro block).

2.0 gm. (4.83 mM) of the dimethyl ester were dissolved in 15 ml. of dry pyridine and 1.4 ml. (14.8 mM) of acetic anhydride were added. The solution, which became reddish brown within a few minutes, was allowed to stand at room temperature overnight. It was concentrated to dryness and traces of the solvents removed by evacuating several hours in a desiccator. The brown crystalline solid was dissolved in 40 ml. of hot benzene, the solution was filtered through a pad of Darco G-60, and 30 ml. of hot hexane (b.p. 60–68°) were added. After standing at room temperature for a few hours and in the refrigerator overnight, the solution deposited a solid mass of light tan rods, melting at 160–161°. A second recrystallization was carried out in exactly the same manner, giving 2.11 gm. (96 per cent yield) of rosettes of white rods (m.p. 162–162.5°, micro block). The analytical sample was dried for 4 hours at 78° over phosphorus pentoxide *in vacuo*.

$C_{23}H_{24}O_8N_2$.	Calculated.	C 60.52, H 5.30, N 6.14
456.14	Found.	" 60.71, " 5.15, " 6.34

Conversion of Dimethyl α,γ-Dibenzamido-β-acetoxyglutarate to Dimethyl α,γ-Dibenzamidoglutarate. Preparation A—2.0 gm. of the β-acetoxy ester were heated in a round bottom flask at 170–190° until the evolution of acetic acid ceased (about 15 minutes). The brown-yellow glass obtained was taken up in 50 ml. of ethanol and reduced in the presence of platinum oxide catalyst at room temperature for 3 hours at 45 pounds of hydrogen pressure. The pale yellow syrup obtained after removal of the platinum by filtration and the ethanol by concentration was hydrolyzed under reflux with 100 ml. of 6 N hydrochloric acid for 5.5 hours. The red-brown solution was taken to dryness and traces of hydrochloric acid removed in a vacuum desiccator by means of potassium hydroxide pellets. To the chocolate-brown salt were added 6 ml. of 2 N sodium hydroxide and 10 ml. of water. After filtration through a pad of Darco G-60, the solution, which was a pale orange-yellow color, was benzoylated by the Schotten-Baumann method with 2 ml. of benzoyl chloride and 20 ml. of 2 N sodium hydroxide in eight equal portions at 2 minute intervals. Upon the addition of concentrated hydrochloric acid, with cooling, to a pH of 2.0, only benzoic acid precipitated. It was removed by filtration. The filtrate was acidified further, concentrated, and allowed to stand overnight in the refrigerator, when a light brown solid separated. Benzoic acid was removed by repeated extractions with hot hexane, leaving 640 mg. of a tan solid. Treatment with Darco G-60 and recrystallization from hot water yielded 300 mg. (19 per cent) of α,γ-dibenzamidoglutaric acid melting at 202–205° (micro block). A second crop of 110 mg. (7 per cent) was obtained from the mother liquors. An analytical sample of this material was prepared by recrystallization from hot water. It was dried at 78° for 6 hours *in vacuo* over phosphorus pentoxide.

$C_{19}H_{18}O_6N_2$.	Calculated.	C 61.61, H 4.90, N 7.57
370.35	Found.	" 61.81, " 4.96, " 7.62

300 mg. of the acid were converted to the dimethyl ester by treatment in the usual manner with an ethereal solution of diazomethane. The white crystalline product obtained melted at 130–165° (micro block), but repeated recrystallizations succeeded in removing the low melting ester. 210 mg. (12 per cent yield) of fine, silky white rods were obtained which melted at 178–179° (micro block) and gave no depression when mixed with a synthetic sample of the high melting dimethyl α,γ-dibenzamidoglutarate (7). An analytical sample was prepared and dried *in vacuo* over phosphorus pentoxide for 5 hours at 78°.

$C_{21}H_{22}O_6N_2$.	Calculated.	C 63.30, H 5.57, N 7.03
398.41	Found.	" 63.37, " 5.63, " 7.26

Preparation B—500 mg. of β-acetoxy ester were heated in a round bottom flask at 170–190° for 30 minutes. The orange-brown glass was reduced in the presence of platinum oxide catalyst at 45 pounds of hydrogen pressure at room temperature for 3 hours. Platinum was removed by filtration and the ethanol by concentration. The glass obtained was crystallized from 10 ml. of hot acetone by adding 40 ml. of hot hexane (b.p. 60–68°). 100 mg. of white silky rods, melting at 155–173° (micro block), were obtained, which after several recrystallizations melted at 178–179° (micro block). This product was identical in every way with the synthetic ester.

The filtrate was reworked with use of a combination of hot acetone and hot hexane as solvents. 50 mg. of starting material were obtained as well as 150 mg. of a mixture of the high and low melting esters which melted at 130–155° (micro block). The mixture was fractionally crystallized, but the low melting ester was not obtained pure. However, individual rosettes of this ester melting at 150.5–152° (micro block) were separated mechanically. When mixed with a synthetic sample of low melting dimethyl

α,γ-dibenzamidoglutarate, these crystals gave no depression of the melting point. A sample of the mixture melting at 133–155° gave correct analytical data for dimethyl α,γ-dibenzamidoglutarate.

$C_{21}H_{22}O_6N_2$.	Calculated.	C 63.30, H 5.57, N 7.03
398.41	Found.	" 63.30, " 5.68, " 7.10

SUMMARY

N,N'-Dibenzoylstreptamine is oxidized by periodate to 2,4,6-trihydroxy-3,5-dibenzamidotetrahydropyran and the latter is oxidized to α,γ-dibenzamido-β-hydroxyglutaric acid by bromine water. The structure of the dibenzamidohydroxyglutaric acid has been confirmed by conversion to the known α,γ-dibenzamidoglutaric acid.

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THE METABOLISM OF DIHYDROXYPHENYLALANINE BY GUINEA PIG KIDNEY EXTRACTS

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Ascorbic acid has been shown to be necessary not only for the metabolism of phenylalanine and tyrosine but also of 3,4-dihydroxyphenylalanine. In the latter connection it has been found that normal guinea pig kidney slices readily oxidize dihydroxyphenylalanine (1), whereas kidney slices from scorbutic animals are almost completely unable to metabolize this amino acid. With the addition of the crystalline vitamin to the deficient slices, dihydroxyphenylalanine oxidation proceeds normally as shown by oxygen consumption and carbon dioxide production.

The difficulty of determining with intact tissue slices the mechanism whereby ascorbic acid produces its effect is obvious. The problem, however, should prove somewhat simpler with cell-free extracts of kidney which would permit fractionation or other manipulations common to enzyme characterization. In this characterization, one may rely upon measurement of gaseous exchange only, as was done previously with kidney slices, but with the possibility that the metabolic reactions produced by kidney enzymes may be more complex, a more complete analysis should afford additional information relative to the entire system. With this possibility in mind, dihydroxyphenylalanine has been incubated with cell-free extracts of guinea pig kidney and the reaction followed by manometric measurements and colorimetric analysis, the latter by the very excellent Arnow method (2). Use of this nitrous acid-molybdate reagent, which measures the catechol value, has led to the finding that the *o*-dihydroxyphenyl portion of the amino acid is involved in the reactions catalyzed by kidney extracts. With these methods a comparison of normal and vitamin C-deficient extracts, an analysis of the over-all reaction, and a characterization of the enzymes involved have been made as described in this communication.

EXPERIMENTAL

Guinea pigs of 300 to 500 gm. were maintained on Purina rabbit chow checkers (complete ration) plus an adequate supply of mixed green food. Those animals used in the investigation of the effect of vitamin C depletion were fed a basal diet of ground and aerated Purina rabbit chow, and half of them received in addition 20 mg. of crystalline ascorbic acid daily. These animals also received 0.9 gm. of Squibb's brewers' yeast every third day and 1 ml. of cod liver oil weekly.

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The enzyme extracts were prepared as follows. The non-fasted animals were stunned by a blow on the occiput and bled by severing the jugular veins. The kidneys were then removed, cleaned, weighed, and placed in an ice-cooled, micro Waring blendor jar with enough cold 0.1 M phosphate (sodium and potassium) buffer to cover the blendor knives and thus prevent excessive splashing. Homogenization was continued, with occasional cooling in the ice bath, until the preparation was essentially homogeneous, and cell-free. The mixture was then centrifuged at 2500 R.P.M. for 5 minutes. The supernatant was removed and the residue reextracted one to three times with the buffer. The combined supernatants were then diluted so that aliquots represented definite quantities of original kidney as indicated subsequently. Just prior to use, the pH was adjusted to the desired value.

The ability of the extracts to metabolize dihydroxyphenylalanine was followed by means of the usual manometric procedure. 1 ml. of the extract and 1 mg. of substrate in a final volume of 2 ml. were employed. After an incubation period of 3 hours, the contents of each vessel were quantitatively transferred with washing into 1 ml. of 10 per cent (1.25 M) metaphosphoric acid contained in a 15 ml. graduated centrifuge tube and the volume adjusted to 10 ml. with distilled water. The contents were thoroughly mixed, allowed to stand for ½ hour, and then the protein precipitate removed by centrifugation. In order to determine the amount of substrate which had been metabolized the centrifugate was analyzed by means of the Arnow colorimetric method for dihydroxyphenylalanine. Readings were made with the Klett-Summerson photoelectric colorimeter equipped with Filter 420. Since the *o*-dihydroxyphenyl compounds are determined by this method, the values obtained have been designated as "catechol" values. In each series of determinations appropriate tissue controls have been included. In order to relate oxygen consumption to the reduction of the catechol value, excess oxygen to substrate ratios have been calculated. In the first place, the oxygen in atoms per mole of amino acid initially present ($O:D_p$) has been calculated. Likewise the ratio of the oxygen consumed to the reduction in catechol value ($O:D_c$) has been determined.

By using these methods a comparison of extracts from normal and scorbutic guinea pigs has been made. In marked contrast to the results obtained with surviving kidney slices (whole cell preparations) (1) normal and scorbutic kidney extracts exhibit no significant difference in oxygen consumption in the presence of dihydroxyphenylalanine. For example, with five preparations from normal guinea pigs the average excess oxygen was 73.1 μl. as compared to 71.3 μl. for five preparations from scorbutic animals, as may be seen in Table I. That the similarity in oxidation is real is confirmed by the values for reduction of catechol content, which are 84.5 and 81.8 per cent, respectively. In turn the similarity is shown by the average oxygen to substrate ratios presented in Table I. In order to confirm the finding that ascorbic acid is not a limiting constituent in the deficient extracts, crystalline vitamin was added to additional control and experimental flasks. As shown in Columns 4 and 5 of Table I, the addition of the vitamin did not alter the final values. The excess oxygen, the results of the colorimetric analyses, and the ratios are for all practical purposes identical.

TABLE I

Dihydroxyphenylalanine Oxidation by Normal and Scorbutic Guinea Pig Kidney Extracts

The values shown are the results of 3 hour incubations at pH 7.4 and at 37.5° with 1 ml. of 10 per cent extract and 1 mg. of substrate in 2 ml. of reaction volume. The values in Columns 2 and 3 are the average values obtained with five guinea pigs, while those in Columns 4 and 5 were obtained with three guinea pigs.

(1)	Normal (2)	Scorbutic (3)	Scorbutic (4)	Scorbutic + ascorbic acid (5)
Basal oxygen, μl.	31.1 ± 1.9	28.6 ± 2.4	29.6 ± 3.0	33.1 ± 2.4
Excess " "	73.1 ± 3.9	71.3 ± 5.0	70.8 ± 4.0	68.7 ± 4.7
Reduction of catechol value, %	84.5 ± 3.40	81.8 ± 1.9	79.8 ± 0.3	78.8 ± 1.5
$O:D_p$*	1.28 ± 0.06	1.22 ± 0.09	1.24 ± 0.07	1.21 ± 0.14
$O:D_e$	1.56 ± 0.03	1.56 ± 0.08	1.55 ± 0.09	1.54 ± 0.17

* See explanation in the text.

Even though the scorbutic animals at the time of sacrificing exhibited the classical symptoms of scurvy, it is well known that an animal dying of vitamin C deficiency still contains appreciable quantities of the vitamin in the tissues. A more rigorous test would be possible if there were some means of causing a more complete depletion of the kidney ascorbic acid. In numerous instances, we have observed that the feeding of extra tyrosine causes a more severe depletion. Therefore, two additional guinea pigs were fed the deficient diet supplemented with 10 per cent tyrosine. On the 8th and 10th days, respectively, these animals showing severe scurvy were sacrificed. The resulting extracts again were found to metabolize dihydroxyphenylalanine as well as normal extracts. As may be seen from Table

II three different concentrations behaved entirely like the corresponding concentrations of the non-deficient extract. Both gasometric and colorimetric analyses show the same picture.

As a consequence of these results, it must be concluded that a state of scurvy in the guinea pig makes no difference in the ability of the subsequent cell-free extract, prepared by the method described, to metabolize the amino acid. In order to analyze this unexpected difference between extract and slice (1) and determine why the two should behave differently with respect to vitamin C deficiency, it was first necessary to analyze and characterize the enzyme reactions and components involved.

TABLE II

Dihydroxyphenylalanine Oxidation by Extracts from Normal Guinea Pigs and Extracts from Guinea Pigs Fed Extra Tyrosine

The incubations were carried out as described in Table I except that the pH was 6.8.

Extract concentration	Oxygen consumption		Catechol compound disappearance
	Basal	Excess	
Guinea pigs fed 10% tyrosine			
per cent	*µl.*	*µl.*	*per cent*
2.5	12.8	24.6	36.0
5.0	17.0	49.5	57.4
10.0	31.5	60.8	74.0
10.0	23.9	56.9	73.4
Normal guinea pigs			
2.5	10.3	27.1	31.1
5.0	17.4	54.1	60.7
10.0	25.8	58.4	71.6

At this point it should be recalled that kidney extracts and in particular extracts made from guinea pig kidneys have for some time been known to metabolize dihydroxyphenylalanine. Originally, it was shown by Holtz in 1938 (3) that the amino acid is decarboxylated and the resulting amine, hydroxytyramine, is readily oxidized by the well known amine oxidase. Subsequent papers by Holtz and associates (4) and by Blaschko and his coworkers (5, 6) have contributed significantly to an understanding of the reactions in question. The extracts used in our own study are quite similar to those employed by the above authors but it remains necessary to show that the systems are the same. Further, it must be determined whether or not the reduction of "catechol" value described above but not previously reported follows decarboxylation and amine oxidation in sequence or whether it is an unrelated reaction.

With this purpose in mind numerous experiments with dihydroxyphenylalanine and also hydroxytyramine as substrates have been carried out. The results obtained demonstrated that the extracts employed in this study decarboxylate the amino acid and oxidize the resulting amine in the same fashion as was described by Holtz and Blaschko. For example, with the amino acid as the substrate and under anaerobic conditions, carbon dioxide evolution was very rapid and for all practical purposes complete within the first half hour. On the other hand, repetition of the same experiment aerobically demonstrated that oxygen was consumed more slowly, the uptake being complete in approximately 3 hours. In the latter experiments, carbon dioxide production, measured at the end of the incubation, was found to be approximately that expected from the other results. The anaerobic carbon dioxide production, as well as the difference in rate of carbon dioxide production and oxygen consumption, almost conclusively proves that oxygen consumption follows decarboxylation of the amino acid. Added evidence is available in the fact that with hydroxytyramine as the substrate the total oxygen consumed and the rate of uptake are essentially the same as those obtained with the amino acid, as is illustrated in Fig. 1.

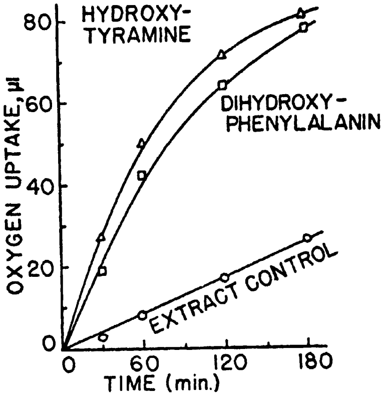

FIG. 1. Oxygen consumption with hydroxytyramine or dihydroxyphenylalanine. With each substrate 5.08 µM in 2 ml. of total reaction volume at pH 6.8 were used and 1 ml. of 10 per cent kidney extract was included.

By employing cyanide which inhibits the decarboxylase but not the amino oxidase (4, 6), it has been found that oxygen consumption is strongly inhibited (53 to 84 per cent by 0.00046 M cyanide) when the amino acid was the substrate. On the other hand, with hydroxytyramine insignificant inhibition of oxygen consumption was observed. An entirely similar picture was obtained when semicarbazide or cysteine was employed as the inhibitor. Consequently, the above conclusion relative to the sequence of reactions is confirmed.

The additional fact, already shown in Table I, that during incubation the dihydroxyphenyl group has disappeared as measured by the catechol value, raises the question as to whether this is also a result of oxidation by the oxygen consumed or the result of some other reaction. The total extra oxygen in numerous experiments has been sufficient to give ratios of 1.2 to 1.4 atoms per mole of substrate found to disappear. This amount of oxygen is only a little more than would be required for the oxidation of the dihydroxy group to the quinone. If the assumption that 1 atom of oxygen is used for this purpose is correct, then it would appear that insufficient oxygen would be left to account for amine oxidation. Therefore, it was important to determine the amount of ammonia formed during the reaction and to relate it to oxygen consumption and reduction of catechol value. The excess ammonia production in a typical experiment was found to be 7.22 μM and the reduction of catechol value 7.63 μM. If we assume 1 atom of oxygen for each reaction, then 14.85 microatoms would be theoretically needed. However, as may be seen in Table III, only 9.81 μM or 65.9 per cent of this amount was actually taken up. However, the rôle of oxygen in amine oxidation has been well established (4, 5), and since sufficient oxygen was not consumed to account for both amine oxidation and the reduction of catechol value, it is reasonable to assume that the disappearance of the dihydroxyphenyl group is not the result of a direct reaction involving atmospheric oxygen.

TABLE III

Oxygen Consumption, Dihydroxyphenyl Disappearance, and Ammonia Formation

Ammonia formation	Catechol compound disappearance	Oxygen consumption		
		Calculated	Found	Found/Calculated
μM	*μM*	*microatoms*	*microatoms*	*per cent*
6.78	7.63	14.41	9.96	69.0
7.42	7.63	15.05	9.68	64.3
7.42	7.63	15.05	9.78	64.8
7.28	7.63	14.91	9.81	65.7
Average 7.22	7.63	14.85	9.81	65.9

This conclusion is also supported by the results obtained by the use of selected enzyme inhibitors. Of the many inhibitors used in an attempt to obtain evidence which would throw light on the relationship of the oxygen consumption to the reduction of the catechol value, cyanide and semicarbazide were the most informative. Since these substances inhibit the dihydroxyphenylalanine decarboxylase reaction, hydroxytyramine was also used as the substrate. In the case of cyanide the results appearing in Table IV demonstrated a marked inhibition of the 3,4-dihydroxyphenyl disappearance (69 per cent) in the presence of a very slight inhibition of the oxygen consumption (27.7 per cent). In the same manner 0.005 M semicarbazide inhibited the dihydroxyphenyl disappearance to the extent of 60.2 per cent, whereas the oxygen consumption was inhibited but 12 per cent.

TABLE IV

Inhibition of Dihydroxyphenylalanine and Tyramine Metabolism

Incubation conditions were as previously described. In the case of cyanide addition, the correct concentration was maintained in the main compartment by addition of a potassium cyanide and potassium hydroxide mixture to the center well (10).

Inhibitor	Concentration	Substrate	Inhibition of	
			Oxygen consumption	Catechol compound disappearance
	mole per l.		*per cent*	*per cent*
Cyanide	0.00046	Hydroxytyramine	27.7	69.6
	0.00046	Dihydroxyphenylalanine	53.3	72.0
Semicarbazide	0.005	Hydroxytyramine	12.0	60.2
	0.05	"	32.3	60.2
	0.05	Dihydroxyphenylalanine	80.7	81.2

For comparison Table IV also contains the results of experiments on cyanide and semicarbazide inhibition in which dihydroxyphenylalanine was the substrate. Although the inhibition of the dihydroxyphenyl group reaction was about the same as when hydroxytyramine was used, the inhibition of the oxygen consumption was greatly increased. When the amino acid was the substrate, the reduced disappearance of catechol value may be interpreted as a result of inhibition of decarboxylation. As a consequence, the later reactions in the chain did not occur. With hydroxytyramine as the substrate, inhibition of decarboxylation is not involved. Nevertheless, strong inhibition of catechol disappearance occurred even though only slight inhibition of oxygen uptake was observed. This discrepancy in amount of inhibition would not have occurred if the oxygen were being used in the *o*-dihydroxy reaction.

The production of hydrogen peroxide in the amine oxidase reaction is well established and many investigators have shown that when this hydrogen peroxide is used to oxidize any easily oxidizable substances in the reaction mixture a total of 2 atoms of oxygen is consumed. Since this total of 2 atoms was not realized in the case under discussion, it may be concluded that the hydrogen peroxide or the oxygen produced by its decomposition was not responsible for the reduction in catechol value. This was confirmed, however, by carrying out the reaction under conditions in which hydrogen peroxide in excess of that produced by the action of the amine oxidase on hydroxytyramine was present during the reaction. This was achieved by carrying out the enzymatic reaction in the presence of tyramine, which, like hydroxytyramine, is a substrate for the amine oxidase and also produces hydrogen peroxide. It may be seen from the results recorded in Table V that the presence of the second substrate resulted in a doubling of the oxygen uptake. In spite of the resultant increased production of hydrogen peroxide there was no increase in the disappearance of the catechol nucleus.

TABLE V

Effect of Simultaneous Tyramine Oxidation (Hydrogen Peroxide Production) on Dihydroxyphenylalanine Metabolism

The incubations were carried out at pH 6.8 and at 37.5° with 10 per cent kidney extracts. 5.08 μM of amino acid and 10.16 μM of tyramine were used as substrates as indicated.

Substrate	Excess oxygen consumption	Catechol compound disappearance
	μl.	μM
Dihydroxyphenylalanine	55.3	3.87
" and tyramine	114.5	3.30

Since from the results of colorimetric analysis it was evident that a reduction of the catechol value did occur, the question of the fate of the aromatic nucleus of the amino acid was an important consideration. With this question in mind the absorption spectra of the metaphosphoric acid centrifugates were determined in order to confirm the above evidence and to determine, if possible, the properties of the end-products of the reactions. For this purpose a Beckman spectrophotometer was used, a minimum slit width being used at all times in order to obtain maximum resolution. The data obtained are illustrated in Figs. 2 and 3, in which optical density is plotted against wave-length. Dihydroxyphenylalanine (Curve I) and catechol (Curve II) exhibit maxima at approximately 2800 and 2750 A. After incubation, the amino acid maximum (corrected for tissue background) at 2800 A has disappeared and a new maximum is evident at approximately 2550 to 2600 A (Curve IV of Fig. 3). Another less pronounced maximum at 3150 A has also appeared.

In an attempt to separate the substance responsible for this new maximum from the metaphosphoric acid filtrates, butyl alcohol was used as an extracting agent. The butyl alcohol was then removed by vacuum distillation under carbon dioxide and the residue dissolved in 0.1 N hydrochloric acid. The ultraviolet absorption spectrum of this extract is represented in Fig. 3. The similarity of this curve with that produced by the unfractionated incubated extract (Curve IV) is at once apparent even though the concentration of the new compound is less. These results are entirely indicative of the elimination or extensive modification of the 3,4-dihydroxyphenyl portion of the amino acid. They support the data obtained with the Arnow analytical procedure and also furnish strong evidence for the production of a new compound with a characteristic absorption spectrum, in the enzymatic reaction.

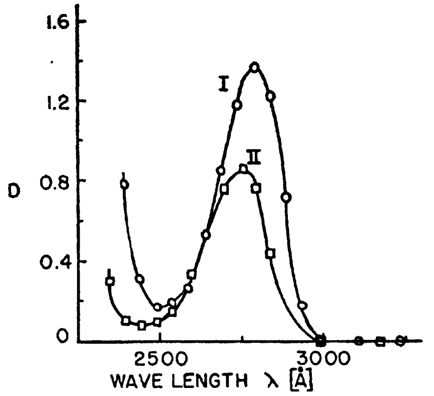

FIG. 2

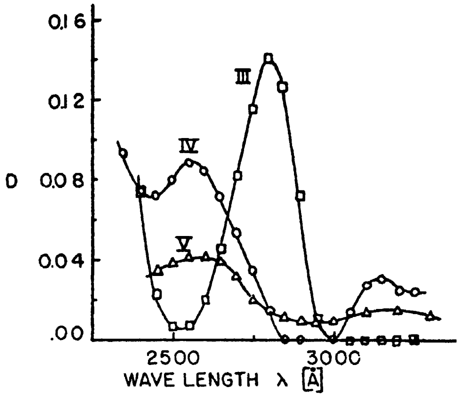

FIG. 3

FIG. 2. Ultraviolet absorption spectra of dihydroxyphenylalanine, Curve I, (0.005 M) and catechol, Curve II, (0.004 M) in 0.1 N hydrochloric acid. 1 cm. cells were used; $D = \log (I_0/I)$.

FIG. 3. Ultraviolet absorption spectra of dihydroxyphenylalanine and the enzyme reaction product. Curve III represents 1 ml. of 0.0025 M amino acid in 0.125 N metaphosphoric acid diluted to 10 ml. with 0.1 N hydrochloric acid. Curve IV represents the reaction product from 5.08 μM of amino acid contained in 0.125 N metaphosphoric acid and diluted 10-fold with 0.1 N hydrochloric acid. Curve V represents the butyl alcohol-soluble material redissolved in 0.1 N hydrochloric acid, each ml. containing the amount derived from 0.0258 μM of original substrate. With each curve, the density plotted is the total density minus that due to the tissue constituents.

Mason (7) followed the oxidation of dihydroxyphenylalanine by "mammalian dopa oxidase" by the change in the ultraviolet absorption spectrum of the reaction mixture, and although he obtained a maximum at 3050 to 3100 A, he did not observe a point of maximum absorption at 2550 to 2600 A. The maximum at approximately 3100 A observed in this study may have resulted from miscellaneous oxidation of the "dopa oxidase" type, but in any case the maximum at 2550 to 2600 A is not comparable with the results obtained in Mason's investigation. Therefore, it may be concluded that the product of the metabolism of dihydroxyphenylalanine by guinea pig kidney extracts is not similar to that obtained in the "dopa oxidase" reaction.

Conjugation of phenol and catechol type compounds is a well known detoxication mechanism and recently DeMeio *et al.* (8) and Bernheim and Bernheim (9) have demonstrated conjugation of phenols by employing the slice technique. Therefore, the metaphosphoric acid filtrates were tested for the presence of conjugates. The method of acid hydrolysis usually employed to hydrolyze catechol conjugates was followed by means of the Arnow analytical procedure. In no case was there encountered any increase of total catechol value, from which it may be concluded that conjugation of the *o*-dihydroxyphenyl group is not involved in the series of reactions under investigation.

DISCUSSION

Results described above show that extracts of kidneys from scorbutic and normal guinea pigs oxidize dihydroxyphenylalanine equally well. This finding is in direct contrast to that obtained with surviving kidney slices, for slices removed from a scorbutic animal are unable to oxidize this amino acid (1). The primary difference in the two cases is that of intact cellular structure which in itself furnishes no real explanation. Nor is an explanation immediately apparent in certain minor points of difference. For example, a relatively greater weight of tissue was present when extracts were employed, as is usually the case when one proceeds from slice to extract technique. This fact does mean that a proportionately greater quantity of ascorbic acid is present in the latter case, for it is well known that a guinea pig dies of scurvy before the tissues are depleted of the vitamin. Consequently, ascorbic acid may have been a limiting factor in the former study. At least, the addition of the crystalline vitamin caused the deficient slices to regain their ability to oxidize the amino acid. In the present study a similar addition was without influence. Likewise, attempts to decrease the ascorbic acid content of the extracts were without effect. This difference between cellular and cell-free preparations, therefore, indicates that the rôle of ascorbic acid in the oxidation of this aromatic amino acid is highly complex and its elucidation must await further experimentation.

In characterizing the enzymatic system present in the extracts employed it has been shown that two well known reactions were occurring. First, there was decarboxylation leading to the production of hydroxytyramine which in turn was deaminated by the amine oxidase. In addition, a third reaction not previously described was observed. This involved the disappearance of the catechol nucleus as measured by the Arnow nitrite-molybdate method and the production of a compound identified by means of an absorption maximum at 2550 A. This third reaction, which occurred without direct use of atmospheric oxygen, represents an additional step of more or less importance in the catabolic handling of the amino acid or its derived amine. The further characterization of the compound produced and the factors affecting its production will be of interest from a purely metabolic standpoint. However, there is another point of interest. Hydroxytyramine is a recognized hypertensive agent. Consequently, the discovery of an additional reaction for the disposal of it and its precursor, dihydroxyphenylalanine, affords a new approach to the problem of essential hypertension. Further analysis of the reaction involving the catechol nucleus should add to a more complete understanding of the chemical phase, particularly that pertaining to the action of the sympathomimetic amines.

SUMMARY

Centrifuged cell-free extracts prepared from scorbutic or non-scorbutic guinea pig kidneys were found to metabolize dihydroxyphenylalanine equally well when oxygen consumption and disappearance of the *o*-dihydroxyphenyl group were measured.

By the appropriate use of the amino acid and hydroxytyramine with aerobic and anaerobic conditions and with selected inhibitors (cyanide and semicarbazide), the nature of the reactions occurring has been determined. The amino acid has been found to undergo decarboxylation and subsequent "amine" oxidation due to the presence of the respective and well known enzymes in the extracts employed.

Both substrates in the presence of the extract underwent a further reaction involving loss of the catechol nucleus without direct mediation of atmospheric oxygen or hydrogen peroxide and without formation of catechol conjugates. This reaction, not previously recognized, was strongly inhibited by cyanide and semicarbazide.

The product resulting from the disappearance of the catechol portion was found to possess an ultraviolet absorption spectrum different from those of the substrates. There was observed one maximum at 2550 A as well as a second less pronounced maximum at 3150 A. This compound was readily separated from acid solution by means of aqueous butyl alcohol.

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A STUDY ON GROWTH INHIBITION BY D-, L-, AND DL-ETHIONINE IN THE RAT AND ITS ALLEVIATION BY THE SULFUR-CONTAINING AMINO ACIDS AND CHOLINE

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Several years ago Dyer (1) reported that ethionine (*S*-ethylhomocysteine), when administered to young rats with a 5 per cent casein diet, induced a rapid loss in weight terminating in death. The simultaneous presence of methionine equivalent to an amount of ethionine in the diet prevented the loss in weight and no deaths occurred during the experimental period. Dyer concluded that ethionine could not be utilized by the rat in lieu of methionine probably because no metabolic deethylation of ethionine to homocysteine took place. Experiments with an amino acid diet supplemented with choline and containing ethionine in lieu of methionine have led to similar conclusions (2). Harris and Kohn (3) observed a growth inhibition effect produced by ethionine in *Escherichia coli* which was completely reversed by methionine in one-tenth the concentration of ethionine in the medium. These experiments furnished the basis for the view that ethionine is an antimetabolite of methionine.

Because of the variety of the metabolic pathways of methionine and of the metabolic products which are known to arise from methionine or from its degradation products (cystine, homocystine, cystathionine, choline, creatine, etc.), it is not at all clear whether the inhibition of growth by ethionine is a result of a "block" in the utilization of methionine *per se*, or whether there is interference with the utilization of some of the metabolites which normally originate from methionine. The present report deals with the inhibition of growth of rats by ethionine on a complete 25 per cent casein diet and with a study of its alleviation by cystine, homocystine, cystathionine, methionine, and choline. The effectiveness of the optical isomers of ethionine in the inhibition of growth and its alleviation by either isomer of methionine is also described.

EXPERIMENTAL

The diet used by Dyer (1) contained 5 per cent of casein. Choline was probably supplied by the vitamin concentrate. On this diet the rats do not grow, although the weight is maintained. Since such a diet is evidently deficient in the sulfur-containing amino acids, and its adequacy in labile methyl groups is uncertain, and since recent reports suggest a probable deficiency of such a diet in other essential amino acids such as lysine, histidine, valine, threonine, and tryptophan (4), it appeared more suitable for our purpose not to complicate the picture by other possible nutritional deficiencies and employ a diet which is adequate for the growth of the rat. We selected a 25 per cent casein diet. As has been shown by Griffith and Wade (5), such a diet is ample in all the essential amino acids, but is somewhat deficient in labile methyl groups. To produce livers with "normal" lipide content 40 per cent of casein was required in the diet. However, for the study of possible labile methyl deficiency in the rat on administration of ethionine, a 25 per cent casein diet appeared quite satisfactory. The percentage composition of the diet was as follows: Labco vitamin-free casein 25, sucrose 15, cornstarch 31, inorganic salts (6) 4, Crisco 20, cod liver oil 5. 1 kilo of this diet contained 10 mg. of thiamine hydrochloride, 10 mg. of riboflavin, 10 mg. of pyridoxine hydrochloride, 20 mg. of nicotinamide, 50 mg. of Ca pantothenate, 5 mg. of folic acid, 1.0 mg. of biotin, 1000 mg. of *p*-aminobenzoic acid, 1000 mg. of inositol, and 7 gm. of liver extract (Lilly). No choline was added to the diet.

Male albino rats of the Wistar strain born and raised in this laboratory were used. They were selected from several litters at the ages of 24 to 35 days. The older rats were used in experiments in which choline deficiency was expected to be more severe. This appeared desirable, as in the course of this work it was found that choline bears a definite relationship to the inhibition of growth by ethionine. The animals were housed in individual cages with raised screened floors, and water and food were allowed *ad libitum*. The food consumption and the changes in weight were recorded twice weekly. All supplements were fed mixed with the diet in amounts indicated in Tables I and II. DL-Ethionine was synthesized according to Dyer (1). D-Ethionine and L-ethionine were similarly prepared from *S*-benzyl-D-homocysteine and *S*-benzyl-L-homocysteine respectively, which were obtained by the resolution of *S*-benzyl-DL-homocysteine (7). L-Ethionine showed a rotation of $[\alpha]_D^{23} = +20.1°$ for a 1 per cent solution in 1 N HCl; for D-ethionine the corresponding value was $-20.4°$. D-Methionine and L-methionine were prepared by the resolution of DL-methionine (8). Both isomers of methionine had an equal and opposite specific rotation of 22° in 1 per cent solution in 1 N HCl. L-Cystine was prepared from hair, inactive homocystine was obtained from methionine (9), and a mixture of L-cystathionine and L-allocystathionine was synthesized by the recently described procedure (10). Choline chloride was a commercial product. All the substances were analytically pure.

At the end of the experiments the kidneys and livers of the rats were examined macroscopically and, if warranted, microscopically. We wish to thank Dr. Clark Brown, the pathologist of the Lankenau Hospital, for the microscopic examination of the tissues. The livers of the rats were weighed immediately upon removal, dehydrated *in vacuo* at room temperature over $CaCl_2$, and the lipides extracted in a continuous extractor with a 1:1 mixture of ethanol and ethyl ether. Upon removal of the solvents the lipides were estimated gravimetrically, and the lipide content was expressed in per cent of fresh liver.

TABLE I

Inhibition of Growth of Rats by DL-*Ethionine and Its Alleviation by Various Supplements Added to 25 Per Cent Casein Diet*

Group No.* (♂)	Initial weight	Final weight	Gain per day	Food per day	Lipides† / Fresh liver	Days on diet	Supplements
	gm.	gm.	gm.	gm.	per cent		per cent
1	79	70	−0.45	3.5	4.9 ± 0.2	20	0.55 ethionine
2	48	84	1.3	3.5	3.7 ± 0.2	27	0.27 "
3	56	143	3.2	5.8	7.4 ± 0.4	27	0.14 "
4	45	158	4.2	7.4	7.3 ± 0.4	27	None
5	44	104	2.1	4.5	6.2 ± 0.3	28	0.55 ethionine, 0.5 methionine
6	45	165	4.3	7.6	4.2 ± 0.2	28	0.5 methionine
7	66	67	0	3.8	4.5 ± 0.2	25	0.55 ethionine, 0.5 cystine
8	54	102	1.92	4.8	4.9 ± 0.3	25	0.55 " 0.5 " 0.5 choline·Cl
9	58	47	−0.4	2.7	6.2 ± 0.4	25	0.55 ethionine, 1.0 cystathionine
10	62	94	1.3	5.8	4.9 ± 0.3	25	0.55 " 1.0 " 0.5 choline·Cl
11	51	105	1.1	4.3		52	0.55 ethionine, 1.0 homocystine, 0.5 choline·Cl
	105	125	0.5	4.0	3.3 ± 0.2	38	0.55 ethionine, 1.0 homocystine
12	36	91	2.3	6.0		20	0.55 " 0.5 choline·Cl
	91	164	2.0	8.0	4.2 ± 0.2	37	0.55 "
13	53	52	−0.1	3.7		10	0.55 "
	52	158	4.4	6.8		24	0.55 " 0.5 choline·Cl
	158	178	2.5	6.3	3.2 ± 0.2	8	0.55 " 0.5 "

* Each group consisted of six rats; the values for weight gain and food intakes are averages.

† Each liver was analyzed separately; the data are mean values, including the standard error of the mean.

Table I summarizes the data obtained on several groups of rats maintained on the 25 per cent casein diet. Increasing amounts of ethionine from 0.14 to 0.55 per cent produced progressively greater inhibition of growth in all rats; 0.55 per cent of ethionine prevented the growth and in the majority of the rats caused a loss in weight. Rats weighing 35 to 40 gm. which ingested 0.55 per cent of ethionine in the diet died within 3 to 4 weeks, and the data were omitted from Table I. Neither cystine, homocystine, nor cystathionine fed together with ethionine alleviated the growth inhibition. When any of these supplements was fed together with choline, or when methionine alone was fed, there was a definite resumption of growth. However, it was found also that choline alone could prevent or alleviate the inhibition effect of ethionine. It will be noted from Table I (Group 12) that 0.55 per cent of ethionine fed to a 160 gm. rat does not completely suppress the growth as it does when fed to a 50 gm. rat (Group 13). It will also be observed that a rate of growth equal to that maintained by rats ingesting the basal diet alone was not attained by feeding either choline or methionine together with ethionine. It is possible that a complete reversal of the inhibition by ethionine could have been secured by varying the ratios of inhibitor to alleviator in the diet. For the moment, however, the interesting point

TABLE II

Inhibition of Growth of Rats by D- or L-Ethionine and Its Alleviation by Either D- or L-Methionine Added to 25 Per Cent Casein Diet

Rat No.* (♂)	Initial weight	Final weight	Gain per day	Food per day	Days on diet	Supplements
	gm.	*gm.*	*gm.*	*gm.*		*per cent*
196	46	40	−0.4	2.4	14	0.5 L-ethionine
	40	84	2.3	4.4	19	0.5 " 0.5 L-methionine
	84	120	4.0	8.3	9	None
198	52	52	0	3.1	14	0.5 L-ethionine
	52	90	2.7	4.8	14	0.5 " 0.5 D-methionine
	90	131	3.0	7.5	14	None
200	53	40	−0.9	2.0	14	0.5 D-ethionine
	40	75	2.3	4.7	15	0.5 " 0.5 L-methionine
	75	121	3.3	8.0	13	None
202	58	51	−0.4	2.8	14	0.5 D-ethionine
	51	74	1.3	5.3	14	0.5 " 0.5 D-methionine
	74	121	3.5	7.7	14	None
204	50	112	4.4	8.0	14	"
	112	99	−0.9	4.5	15	0.5 L-ethionine
	99	143	3.4	7.3	13	None
206	59	128	4.9	8.8	14	"
	128	120	−0.5	5.4	15	0.5 D-ethionine
	120	180	4.6	9.0	13	None

* Each rat represents experiments obtained on two animals.

seems to be that choline alone can alleviate the growth inhibition by ethionine. The severity of the inhibition by ethionine seems to be related to the previous administration of choline to young rats (compare data on Groups 11, 12, and 13, Table I, and Rats 204 and 206, Table II).

Table II shows that either isomer of ethionine inhibits the growth of the rat on a 25 per cent casein diet, and that either isomer of methionine alleviates the inhibition by either isomer of ethionine.

None of the rats which were fed ethionine alone or together with the supplements developed fatty livers or showed kidney damage within the period of experimentation.[1] On the contrary, the lipide content of the livers of rats ingesting ethionine alone or in combination with supplements, such as cystine, was lower than that shown by rats ingesting the basal diet. That our diet was somewhat low in labile methyl groups is indicated by the fact that addition of 0.5 per cent of methionine lowered the liver lipide content from 7.3 to 4.2 per cent without appreciably affecting the growth rate (Groups 4 and 6, Table I).[2] It is apparent, therefore, that the inhibition of growth by ethionine does not interfere with the lipide turnover in the liver on a diet which is somewhat low in labile methyl groups.[3]

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[1] In experiments in which rats were maintained continuously on a 25 per cent casein diet supplemented with 0.55 per cent of DL-ethionine and either 0.5 per cent of DL-methionine or 0.5 per cent of choline chloride for 90 days, the rats gained an average of 120 gm. in weight (the initial weight was about 50 gm.). On autopsy, however, the livers of these rats showed no fatty infiltration, but bile-duct proliferation, early fibrosis, and lymphocytic infiltration could be observed. The kidneys showed tubular degeneration, calcification, and infiltration of lymphocytes.

[2] Griffith and Wade (5) observed about 23 per cent of liver lipides with 80 per cent incidence of kidney lesions in rats which were fed a diet containing 25 per cent of casein for a period of 10 days. Our rats showed livers with only about 7 per cent lipides after 27 days of maintenance on our 25 per cent casein diet. Our diet contained corn-starch in addition to sucrose, while that of Griffith and Wade (5) contained only sucrose. It is possible that the corn-starch of our diet contained sufficient choline to offer some protection to the rats against kidney lesions, but not enough to give livers with "normal" lipide content.

[3] In a recent paper Hardwick and Winzler (11) reported that 0.8 per cent of DL-ethionine in an 18 per cent arachin-diet fed to young rats for 12 days caused a severe loss in weight. The rats ingesting ethionine showed less lipides in the liver than those ingesting homocystine or homocystine together with choline in the basal diet. Addition of methionine in amounts equivalent to those of ethionine in the basal diet resulted in alleviation of the inhibition effect of ethionine, but the lipide content of the livers of these animals was over 200 per cent of those ingesting ethionine alone. It should be noted that Griffith emphasized on many occasions that gain in weight in a young rat is a prerequisite for fatty accumulation in the liver and kidney damage on diets low in the labile methyl group.

DISCUSSION

We cannot describe the exact mechanism which is at play in the inhibition of growth by ethionine and its alleviation by either methionine or choline. Apparently the mechanism concerns in some way the labile methyl groups or the intact choline molecule. If the absence of fatty infiltration of the livers of rats ingesting ethionine is the result of choline synthesis from the methyl group of dietary methionine, then apparently ethionine does not interfere with the transmethylation of methionine. The ethionine effect is not one of interference with the synthesis of cystine from methionine, since neither cystine, homocystine, nor cystathionine alleviated the inhibition produced by ethionine. The alleviation of the inhibition by choline suggests, however, that ethionine induces increased demand for choline, presumably at the expense of dietary methionine. That choline might exert a "methionine sparing" effect in the rat, releasing thereby more methionine for growth purposes, has been suggested, and growth stimulation by choline under such conditions has been noted (12). It has also been proposed that the requirement for methionine in the rat is determined by the needs for growth and those for lipotropism (13). It is possible, therefore, that an increased requirement for choline in our experiments which was induced by ethionine administration curtailed the amount of methionine available for growth.

This increase in the need for choline induced in the rat by ethionine ingestion suggests the possibility that ethionine interferes with some processes in which choline *per se* or only the labile methyl group plays a rôle which is essential for normal tissue synthesis. Normally, on a choline-free diet, the labile methyl group of methionine is not exclusively used for the synthesis of choline. A considerable fraction of it is oxidized to CO_2 (14), which in turn may be used for other synthetic reactions. Also there are other essential methylation processes which draw upon the labile methyl group of methionine (15). It is conceivable that the increased need for choline which is created by ethionine administration results in a diversion of the labile methyl group of methionine for greater synthesis of choline. This would tend to create a stress in relation to other methylation reactions, some of which are essential for the well being of the animal. This stress can be rectified by the administration of either methionine, choline, or, perhaps, other methyl donors. It would be of interest to ascertain whether in the presence of ethionine in the diet the methylation to yield creatine, anserine, epinephrine, etc., is diminished in the rat, and by the use of tracer technique to establish whether under these conditions the labile methyl group of methionine is available for various methylation reactions, including the synthesis of choline, to the same extent as in the absence of ethionine.

The observation that either optical isomer of ethionine induced growth inhibition suggests the probability that the D isomer was inverted to the L enantiomorph *in vivo*, probably via the intermediary α-keto acid. In the rabbit, the administration of racemic ethionine leads to an increased excretion in the urine of α-keto acids (16). The activity of the two enantiomorphs of ethionine in the rat is in contrast with the results obtained with β-2-thienylalanine in *Saccharomyces cerevisiae*, *Escherichia coli*, and *Lactobacillus delbrueckii* LD5 (17) and with methoxinine in *Escherichia coli* (18). Only the L isomer of thienylalanine inhibited the growth in all three organisms, and in the case of methoxinine only the L form of methionine alleviated the inhibition induced by the racemic compound. It is also of interest that in the rat methoxinine inhibited growth without producing fatty infiltration in the liver (19). The differences in the response to the two enantiomorphs of antimetabolites in bacteria, as contrasted to those in the rat, may be due to species characteristics, reflecting their ability to utilize only one optical form of an amino acid. Both forms of methionine are available to the rat for synthetic reactions, and it is, perhaps, not surprising that in this animal either optical form of ethionine inhibited growth.

In discussing the relationship of amino acid analogues to protein synthesis in bacteria, Work and Work (20) expressed an idea which appears of interest to the discussion of the possible relationship of ethionine to protein synthesis in the rat. They stated: "To upset protein synthesis, the organism might be presented with amino acid analogues capable of being built up in peptide linkage but incapable of proper function in the completed protein." This idea suggests the possibility of incorporation of ethionine into the tissue protein of the rat. As we stated before, the alleviation of the inhibition by ethionine is not completely reversed by either choline or methionine under the conditions of our experiments, and prolonged administration of ethionine together with either choline or methionine produced pathological changes in the liver and kidney of the animal.[1] In the case of methoxinine its "toxicity" to the rat is still apparent in spite of the beneficial effects of methionine (19). These "subtle effects" (19) of amino acid antimetabolites in the rat warrant further study of their possible relationship to protein synthesis. Employing the tracer technique, we are attempting at present to elucidate the problems which we outlined in the "Discussion."

SUMMARY

1. L-Ethionine, D-ethionine, or DL-ethionine inhibited the growth of rats maintained on a 25 per cent casein diet. L-Methionine or D-methionine alleviated the inhibition by either isomer of ethionine.

2. Neither cystine, homocystine, nor cystathionine alleviated the growth inhibition by ethionine. Choline was effective.

3. No fatty infiltration in the liver or kidney damage were observed in rats ingesting ethionine during the experimental period. Feeding of ethionine over a period of several months together with choline or methionine resulted in liver and kidney damage without fatty infiltration. In the amounts fed, neither choline nor methionine fed together with ethionine stimulated the growth to the level obtained on the basal diet.

4. The possible significance of these results in relation to labile methyl group economy and to tissue protein synthesis in the rat is discussed.

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ON THE AVAILABILITY OF THE DIKETOPIPERAZINES OF CYSTATHIONINE, HOMOLANTHIONINE, AND METHIONINE TO RATS FOR GROWTH PURPOSES

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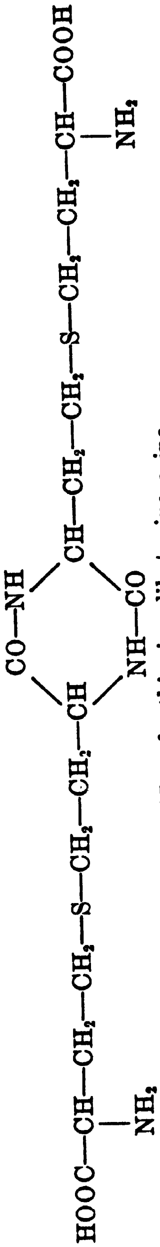
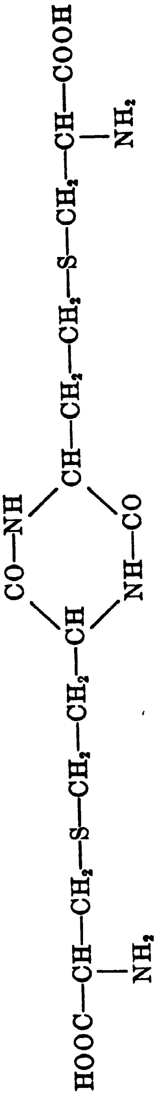
We recently reported the synthesis of cystathionine and of the next higher homologue of lanthionine, homolanthionine (1). The intermediates in this synthesis were the corresponding diketopiperazine derivatives. Inspection of the structures of these intermediates reveals the fact that the cysteine moiety of the cystathionine molecule in the cystathionine diketopiperazine derivative and, similarly, one of the homocysteine moieties of homolanthionine in the homolanthionine diketopiperazine derivative are free. The possibility that these free residues of cystathionine and homolanthionine diketopiperazines might undergo cleavage *in vivo*, and thereby become available to rats for growth purposes, could not be dismissed *a priori*. The structural formulas of the diketopiperazine derivatives of cystathionine, homolanthionine, and methionine are given below. The last compound was included in the study because of the possibility that methionine anhydride sulfur, following the demethylation of the compound *in vivo*, could be linked to the carbon residue of serine, thus giving rise to cystathionine diketopiperazine. Should such demethylation of methionine anhydride occur, the lipotropic effect of the methyl group could then be detected in the rat which is maintained on a diet with low labile methyl group content.

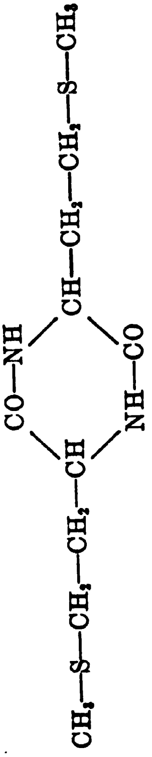
Previous investigations have shown that rat liver preparations cleave L-cystathionine to cysteine (2). L-Cystathionine is available to the rat for growth purposes in lieu of cystine, while L-allocystathionine in the presence of dietary choline stimulates the growth in lieu of methionine (3). D-Cystathionine and D-allocystathionine were neither cleaved to cysteine or homocysteine by rat liver preparations (2) nor were they available to the rat for growth purposes in lieu of cystine or methionine (3). Thus, the *in vivo* and *in vitro* cleavage of cystathionine obviously depended on the optical configuration of the cysteine moiety of the cystathionine molecule, the L form conferring the biological activity on the thio ether. Since our preparation of cystathionine was a mixture of L-cystathionine and L-allocystathionine (both forms being biologically active), the above experimental findings further strengthened the possibility that the cysteine



Homolanthionine diketopiperazine



Cystathionine diketopiperazine



Methionine anhydride

moiety of cystathionine diketopiperazine might be available to the rat by simple cleavage of L-cysteine.

We have recently reported that our preparation of homolanthionine (a mixture of DL and meso forms) is biologically active in lieu of cystine and, to some extent, in lieu of methionine (4). Furthermore, tracer studies have shown that the sulfur of homolanthionine is available for the synthesis of hair cystine in the rat (5). If this activity of homolanthionine was a result of a simple cleavage of homocysteine, then by virtue of the reasoning outlined above for cystathionine diketopiperazine one could expect such cleavage to occur *in vivo* in the case of the diketopiperazine derivative of homolanthionine as well. On the other hand, if the activity of the enzymatic systems involved in the cleavage of either cystathionine or homolanthionine depends not only on the optical configuration of the cysteine

TABLE I

Availability of Diketopiperazines of Cystathionine, Homolanthionine, and Methionine to Rats for Growth Purposes on Low Casein Diet

Rat Group No.*	Initial weight	Final weight	Gain per day	Days on diet	Food per day	Supplements to Diet C-8
	gm.	*gm.*	*gm.*		*gm.*	*per cent*
1	57	86	1.0	28	8.0	0.9 homolanthionine diketopiperazine
	86	90	0.8	5	6.8	None
	90	154	2.7	24	10.5	0.55 homocystine
	154	149	−0.7	7	8.8	None
2	50	71	0.9	24	7.5	0.2 choline·Cl
	71	98	0.9	30	8.0	0.2 “ and 0.9 homolanthionine diketopiperazine
	98	116	1.2	14	9.2	0.2 choline·Cl
3	54	77	0.92	25	6.6	0.85 cystathionine diketopiperazine
	77	132	2.2	25	8.5	0.5 cystine
4	56	81	1.08	23	7.5	0.2 choline·Cl and 0.85 cystathionine diketopiperazine
	81	111	1.0	30	8.3	0.2 choline·Cl
5	48	67	1.1	17	8.0	None
	67	80	0.81	16	8.1	0.6 methionine anhydride
	80	136	1.93	29	9.8	0.61 “
	136	132	−0.5	8	7.4	None
6	55	80	2.5	10	9.2	0.93 cystathionine
	80	95	0.72	21	6.7	None
	95	140	2.8	16	9.8	0.93 cystathionine
7	50	140	1.1	84	8.8	None
8	60	144	1.0	84	9.0	0.2 choline·Cl

* Each group of rats consisted of three animals. The data are average values.

and homocysteine moieties respectively, but also on the availability of the α-amino and carboxyl groups of the rest of the respective molecules, then no stimulation of growth could be expected to result on administration of either of the diketopiperazines. It appears well established that the diketopiperazine bond cannot be split by the animal organism. The study presented here describes the results of testing these alternatives.

EXPERIMENTAL

Five litters of male albino rats of Wistar strain born and raised in this laboratory were used. The experimental conditions employed and the composition of the diet used were previously described (4). The liver lipides of the rats which ingested methionine anhydride were estimated gravimetrically by extraction of the dehydrated liver in a continuous extractor with a 1:1 mixture of ethanol and ethyl ether. The lipide values were expressed in per cent of fresh liver. Cystathionine, cystathionine diketopiperazine, and homolanthionine diketopiperazine were synthesized according to a recently described procedure (1). Methionine, methionine anhydride, and homocystine were prepared according to Snyder *et al.* (6). The compounds were analytically pure. The amounts of the supplements fed to the rats are indicated in Table I.

DISCUSSION

As is apparent from the data presented in Table I, the diketopiperazine derivatives of cystathionine, homolanthionine, or methionine did not stimulate the growth of rats which were maintained on an 8 per cent casein diet. Addition of choline to the diet did not alter the results. On the other hand, administration of either cystathionine, cystine, methionine, or homocystine promptly resulted in stimulation of growth. The preparation of cystathionine synthesized by our method is thus physiologically active, as was expected on theoretical grounds (1). It is apparent from these results that blocking the homocysteine moiety of cystathionine by the diketopiperazine ring abolishes the physiological activity of cystathionine as far as the stimulation of growth is concerned. This could presumably mean that the cysteine moiety of the diketopiperazine derivative of cystathionine, although free, cannot be cleaved by the rat organism. The same conclusion is applicable to the results obtained with homolanthionine diketopiperazine. It could be presumed on the basis of these results that rat liver preparations will be unable to cleave either the cysteine or the homocysteine moieties from the diketopiperazine derivatives of cystathionine and homolanthionine respectively,[1] if such cleavage parallels the biological activity of the compounds *in vivo*.

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[1] Under the conditions which readily permit rat liver preparations to cleave cysta-

The livers of rats which ingested 0.6 per cent of methionine anhydride with the 8 per cent casein diet for 3 weeks contained 21.0 per cent of lipides (average value for six animals). The control animals kept on the basal diet alone for 3 weeks showed 20.0 per cent of lipides in the liver. It is apparent that the methyl group of methionine anhydride was not available to the rat for lipotropism.

SUMMARY

The diketopiperazine derivatives of cystathionine, homolanthionine, and methionine did not stimulate the growth of rats maintained on a low casein diet, in spite of the fact that the cysteine and homocysteine moieties of the diketopiperazines of cystathionine and homolanthionine are free. Methionine anhydride did not lower the lipide content of the livers of rats, suggesting that no demethylation of methionine anhydride took place *in vivo*.

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thionine to cysteine (2), homolanthionine was not cleaved either to homocysteine or to H_2S. Neither was cysteine formed under these conditions in the presence or absence of NaCN (Stekol, J. A., and Weiss, K., unpublished data).

ISOLATION AND PROPERTIES OF CRYSTALLINE α-AMYLASE FROM GERMINATED BARLEY*

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The isolation of crystalline α-amylase has been reported in a preliminary communication (1). Details of the method of isolation, behavior of the fractions leading to crystallization, and some properties of the crystalline material are reported here. This material constitutes the first instance of the crystallization of an α-amylase from a higher plant and is probably the only protein crystallized from barley malt. The only other amylase crystallized from a higher plant is the β-amylase protein obtained from the sweet potato by Balls, Walden, and Thompson (2).

Methods of Assay

Amylase and Other Enzymes—When α- and β-amylases were both present, each enzyme was determined by the methods of Olson, Evans, and Dickson (3). After the β enzyme had been destroyed (by heat) in the course of purifying α-amylase, the α-amylolytic activity was determined as described by Schwimmer (4), by measuring the rate of change in the intensity of the color formed when iodine is added to the digesting starch. Under controlled conditions, the time required to arrive at any specified intensity is inversely proportional to the amount of α-amylase. This proportionality holds over a wide range of enzyme concentrations and of color intensities, but measurements are best when the system transmits about half the incident light.

For reasons of convenience and somewhat increased accuracy, a new unit of α-amylase will be used here and in later communications. The new unit is defined as that amount of enzyme which (with starch as the initial substrate) gives 50 per cent transmission at 660 mμ in 10 minutes under the previously specified conditions.[1] The units may be read off by interpolation on an ordinary curve of time *versus* per cent transmission, made with any preparation of malt α-amylase that has previously been heated to 70°

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* Enzyme Research Division Contribution No. 121.

[1] 1 α-amylase unit as described here is equivalent to 0.16 of the unit of Sandstedt, Kneen, and Blish (5), and to 0.0026 mg. of protein nitrogen in the form of the twice crystallized protein described here.

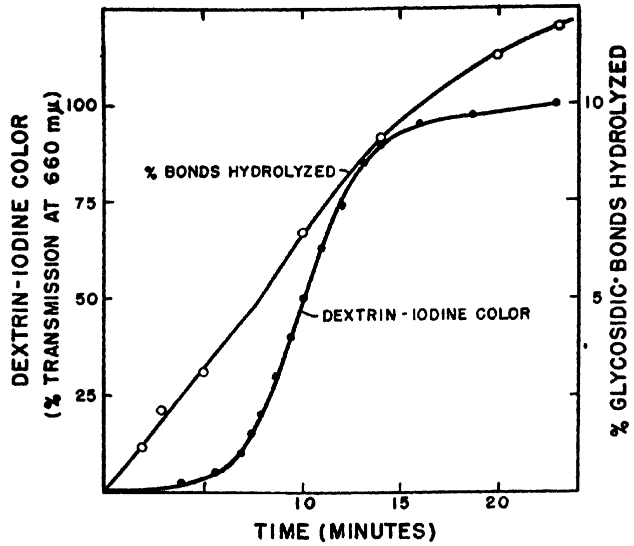

FIG. 1. The change in color of the dextrin-iodine complex and the per cent of glucosidic bonds hydrolyzed as the hydrolysis by α-amylase proceeds.

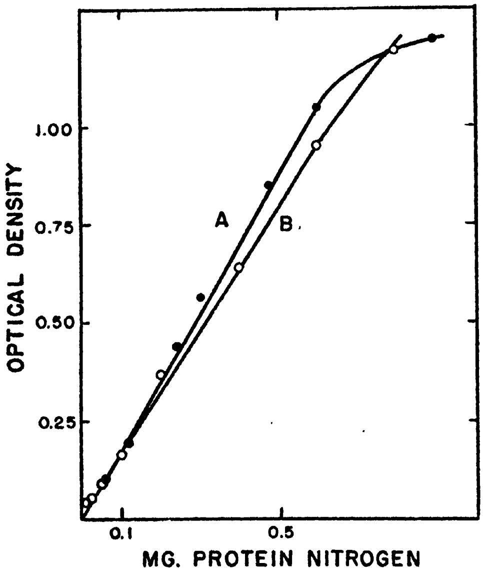

FIG. 2. Relation between optical density of malt protein solutions and protein nitrogen content as determined by micro-Kjeldahl. Curve A, optical density of turbid solutions determined at 400 mμ in an Evelyn colorimeter. The solutions were prepared by mixing 1.0 ml. of a (suitably diluted) crude protein solution with 10.0 ml. of 2 per cent trichloroacetic acid. Concentration is expressed as mg. of nitrogen per ml. of the diluted protein solution before addition of the trichloroacetic acid. Curve B, optical density of 1 cm. of solutions of crystalline α-amylase read at 281 mμ in a Beckman spectrophotometer. Protein nitrogen concentration is expressed as mg. per 10 ml. of solution.

for 15 minutes to free it from the β enzyme. Such a curve is shown in Fig. 1. For comparison the fraction of total glycosidic bonds hydrolyzed in the same time is shown.

Hydrolysis of glycosidic bonds was followed by a modification (6) of the Willstätter-Schudel reduction of hypoiodite (7). This method was also used to determine maltase activity. Phosphatase was assayed by the method of Axelrod (8), which is based on the rate of hydrolysis of *p*-nitrophenyl phosphate.

Protein Nitrogen and Specific Activity—The specific activity is expressed as α-amylase units per mg. of protein nitrogen, [α u./PN]. Protein nitrogen was estimated by one of three methods. For routine analysis of crude preparations a turbidimetric measurement of the precipitate with trichloroacetic acid was made in an Evelyn colorimeter at 400 mμ. Protein nitrogen in colorless, purified preparations was determined by the ultraviolet absorption of the protein solution at 281 mμ measured by a Beckman spectrophotometer. The relation between optical density and protein nitrogen in these two cases is given by Fig. 2. These methods were checked by usual micro-Kjeldahl determinations on washed trichloroacetic acid precipitates.

Procedure for Isolation

Characteristics of Crude Concentrate—Commercial malt syrup was used as source material.[2] This extract was very high in sugar and contained salt and sulfite as preservatives. Its activity was about 1.5 times that of the same weight of a highly diastatic malt; however, the specific activity of the α-amylase therein was 3 to 4 times as high. When heated to 70° for 16 minutes, over half of the protein, including all of the β-amylase, was removed, but there was no loss of α-amylase activity (Table I). In contrast to dilute malt extracts (4), α-amylase cannot be removed from this material by bentonite. No additional calcium salt was required to stabilize the enzyme during heating. As a routine procedure, 2 liters of this concentrate were heated to 70° for 15 minutes and then filtered on a Büchner funnel with 60 gm. of Celite. The residue was then washed with calcium sulfate solution until the final volume was again 2 liters. This filtrate is Line 2 in Table II, where the entire procedure is summarized and the specific activity and yield are shown at each step.

Precipitation by Ammonium Sulfate—Practically all the α-amylase was precipitated from the filtrate just described by making it 0.43 to 0.50 saturated with ammonium sulfate, and adjusting the pH to 5.6 to 6.0 with ammonia.[3] After 2 hours at room temperature, the precipitate was filtered onto fluted paper (Schleicher and Schüll, No. 588), and washed with 5 or 6 times its volume of 0.33 saturated ammonium sulfate solution (pH 5.6 to 6.0) containing 2 gm. of calcium sulfate per liter. The washed precipitate is Line 3 in Table II.

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[2] Obtained through the courtesy of Dr. Alexander Frieden of the Pabst Brewing Company, to whom the authors express their best thanks.

[3] Solid ammonium sulfate was used in the amount required if the liquid was water (instead of being a strong solution of maltose).

This precipitate may be considerably purified by repeated fractional precipitations with ammonium sulfate. A specific activity of 340 was once obtained in this way, to be compared with 390 found later for the crystalline protein. The yields were invariably low, and the material never got beyond the "globular stage," but much information of subsequent value was obtained by studying these preparations. The enzyme exhibited a minimum solubility in dilute ammonium sulfate (0.28 saturated) in the neighborhood of pH 6.0. Furthermore, more protein impurities remained in solution when precipitation was made at pH 6.0 to 7.0 than otherwise.

TABLE I

α-Amylase and Protein Nitrogen in Crude Malt Extract after Heating at 70° and Filtering

Time of heating	Activity $\left[\frac{\alpha\text{ u.}}{\text{ml.}}\right]$	Protein nitrogen	Specific activity $\left[\frac{\alpha\text{ u.}}{\text{PN}}\right]$
min.		*mg. per ml.*	
0	16.0	2.35	6.7
4	16.6		
7	15.9	1.41	11.3
10	15.8	1.25	12.8
16	16.0	1.10	14.6
24	14.0	1.07	13.1
35	13.0	0.95	13.7

TABLE II

Summary of Isolation of Malt α-Amylase

	Volume	Activity per ml. $\left[\frac{\alpha\text{ u.}}{\text{ml.}}\right]$	Total activity $\times 10^3$ [α u.]	Specific activity $\left[\frac{\alpha\text{ u.}}{\text{PN}}\right]$
	ml.			
1. Crude malt extract	2000	14	28	6
2. Heated to 70° and filtered	2000	13.5	27	13
3. Ppt. at 0.43 saturated ammonium sulfate	(17 gm.)		22	80
4. Alcoholic solution of partly purified enzyme	200	95	21	105
5. Combined eluates			17	310
6. Ppt. from eluate			16	
7. Suspension of 1st crystals	30	467	14	350
8. " of crystals, 4 times recrystallized	20	400	8	390

Thus the specific activity of the precipitate obtained at 0.28 saturation of a partially purified enzyme solution that contained 81 units per ml. was 210 at pH 5.1, 260 at pH 6.0, 240 at pH 7.0, and 205 at pH 7.5.

An attempt was also made to predict the specific activity of the pure enzyme by the method of Falconer and Taylor (9). To this end, a solution of partly purified enzyme was precipitated at different concentrations of ammonium sulfate. At zero ammonium sulfate (*i.e.*, when diluted with water instead) the solution contained 135 units of α-amylase per ml. with a specific activity of 197 units per mg. of protein nitrogen. Fig. 3, *b* is plotted from the solubility data of Fig. 3, *a*. According to Falconer and Taylor (9) the tangent *DB* represents the behavior of a system in which the enzyme is pure. Therefore, in the preparation used, 48 per cent of the total protein is inert, and the specific activity of the remaining 52 per cent is 197 × 1.93, or 380. This is in remarkably close agreement with the maximum activity, 390, found for the material crystallized later. The activity and total protein content of the soluble phase are shown in Fig. 3, *a*. The enzyme is seen to be less soluble than the remainder of the protein.

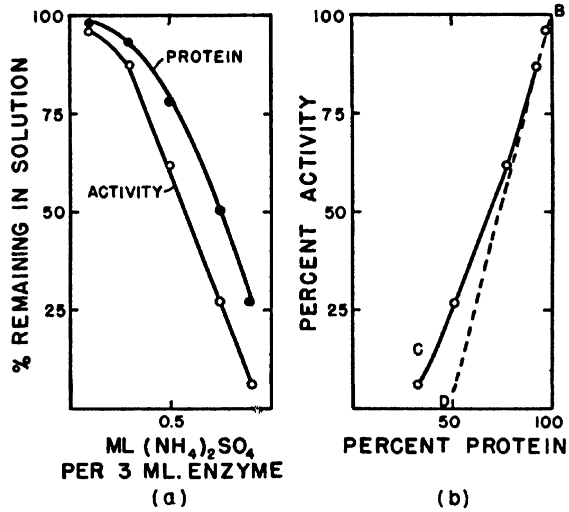

FIG. 3, *a*. Solubility of protein and solubility of the α-amylase activity at several concentrations of ammonium sulfate; pH 5.8; for other data see the text.

FIG. 3, *b*. Activity remaining in solution. Variation of activity with total protein remaining in solution as calculated from Fig. 3, *a*. The slope of the tangent to *CB* is 1.93.

The stability of the partially purified protein toward methanol, ethanol, and acetone was tested. Of these reagents, ethanol seemed to be least destructive. In 40 volume per cent ethanol about half the enzymic activity was lost in 6 hours at 25°, while at 5° no loss whatever occurred in

24 hours. The enzyme was found to be quite soluble in ethanol solutions up to 55 volumes per cent, at temperatures around −20°.

Adsorption on Starch Granules—The adsorption of amylase on starch was reported by Starkenstein in 1910 (10). In 1933, Holmberg (11, 12) found that the α component of malt amylase could be separated from the β component by adsorption of the former on starch, but apparently this method was not used extensively. Hockenhull and Herbert (13) found that the amylase of *Clostridium acetobutylicum* is adsorbed onto potato starch in the presence of alcohol at low salt concentrations.

It was found, starting with material corresponding to Line 3, Table II, that the α-amylase could be adsorbed to some extent on raw wheat starch from aqueous solutions, but much more completely from solutions containing ethanol. Unlike most adsorptions, that of the amylase required considerable time. In one experiment, 100 cc. of 40 per cent ethanol containing 10,500 units of enzyme was stirred at 0° with 20 gm. of raw starch. In 30 minutes, 50 per cent of the activity was adsorbed, 65 per cent in 80 minutes, and 70 per cent (the maximum) in 90 minutes. Some non-selective adsorption of protein also took place. Better results were obtained by the use of a Zechmeister column, usually 40 mm. in diameter, filled to a depth of 10 to 12 cm. with a mixture of equal weights of Celite and wheat starch. The column was used as follows:

The washed precipitate (Line 3, Table II) was made up to a volume of 50 ml. in water; then an equal volume of cold 80 per cent ethanol containing 5 gm. of calcium chloride per liter was added. The insoluble material was filtered out with a little Celite and washed with diluted alcoholic calcium solution (40 per cent alcohol) until the total volume of filtrate and washings was 200 ml. (Line 4, Table II). This was kept for 30 to 60 minutes at +5° and filtered again if a precipitate ($CaSO_4$) appeared. It was poured onto a dry well packed column of starch and Celite and sucked through. The column was usually kept at room temperature. It was next washed with diluted (40 per cent) alcohol until the outflow was colorless. Elution was made by pouring 50 ml. portions of water saturated with calcium sulfate through the column at room temperature. Each portion of eluate was kept separate until assayed and only those containing much enzyme were combined and used (Line 5, Table II).

An experiment was made to determine the distribution of enzyme in the column after the washing procedure. The data are given in Table III. Most of the enzyme remained near the top, but the enzyme toward the bottom was somewhat purer.

Final Purification—The enzyme was precipitated from the combined eluates (about 150 cc.) by 0.66 saturation with ammonium sulfate (pH 6.0). The precipitate (Line 6, Table II) was collected in a little Celite on a small

Büchner funnel, and washed thereon with about 20 ml. of half saturated ammonium sulfate (pH 6.0). It was then dissolved while on the filter in as small a volume as convenient (15 to 20 ml.) of half saturated calcium sulfate. At this stage the enzyme solution was clear, colorless, and contained about 2 to 3 mg. per ml. of protein nitrogen. The pH of the solution was adjusted, when necessary, to 5.9 to 6.0 with 0.1 N ammonia, and saturated ammonium sulfate solution at the same pH was added slowly up to 0.26 saturation. When placed at 30–33°, crystals of the enzyme appeared in 1 to 3 hours. Later, ammonium sulfate was added to 0.33 saturation, and after standing overnight in the incubator the suspension was centrifuged (Line 7, Table II). For recrystallization, the sedimented crystals were taken up in 15 ml. of cold half saturated calcium sulfate solution and the crystallization carried out as before. The protein seems to be more soluble in the cold. Crystal formation was not observed below 25° or below pH 5.6. The specific activity and recovery of enzyme through four successive crystallizations are shown in Table IV. The purity of the mother liquors gradually approached that of the crystals which separated

TABLE III

*Distribution of α-Amylase Activity in Starch-Celite Column after Washing**

Portion No.	Length	Per cent of total activity	Per cent of total protein nitrogen	Relative purity	Per cent activity per cm. of depth
	cm.				
1	0.8	12	13	92	15
2	3.2	42	45	93	13
3	4.6	35	32	110	8
4	1.8	11	10	110	6

* The column was divided into four portions, No. 1 being the topmost. Portions 1 and 3 are slightly higher in Celite content, since a pad of Celite was placed at either end.

TABLE IV

Yield and Specific Activity of Malt α-Amylase on Recrystallization

No. of crystallization	Suspension		Supernatant therefrom	
	Total activity [α u.]	Specific activity $\left[\frac{\alpha \text{ u.}}{\text{PN}}\right]$	Total activity [α u.]	Specific activity $\left[\frac{\alpha \text{ u.}}{\text{PN}}\right]$
1st	30,500	345	6300	273
2nd	22,100	388	1540	298
3rd	19,300	396	1720	340
4th	15,800	389	1270	366

from them. Analysis (Kjeldahl) of the twice crystallized material gave 13.4 per cent nitrogen after prolonged dialysis against distilled water.

The crystals (Fig. 4) are hexagonal prisms, about 13 μ in length, capped by pyramids. Larger crystals (about 30 μ in length) have been occasionally obtained when an amorphous precipitate appeared at first and did not crystallize for several hours. They were usually accompanied by large amounts of amorphous protein.

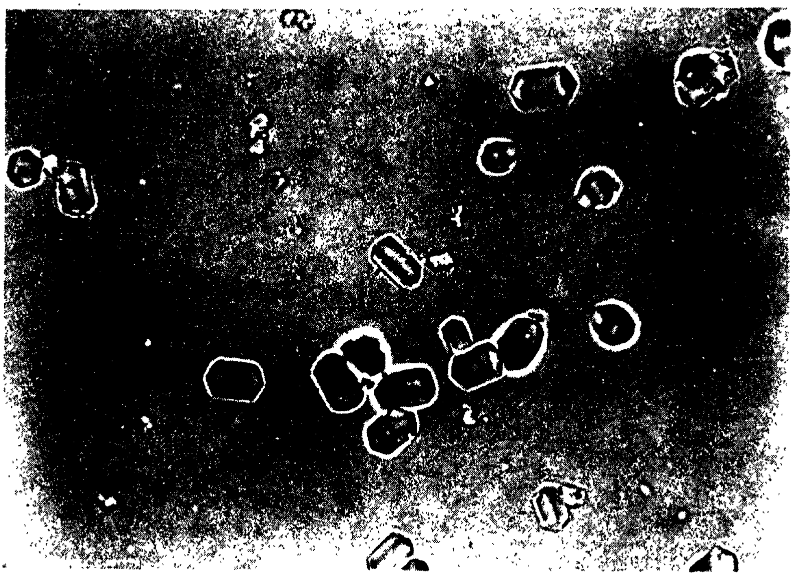

FIG. 4. Crystals of the α-amylase of barley malt (× 580)

Several Properties of Crystalline Protein

Solubility Curve—The solubility of the crystalline protein in half saturated ammonium sulfate (adjusted to pH 5.95 with ammonia) was determined in the presence of increasing amounts of the crystals, according to the technique described by Herriott (14). The crystals were washed with the solvent and then suspended in it. The suspensions were diluted to constant volume with more solvent and the containers were slowly rotated on a horizontal axis for 16 hours at 25°. Thereafter the solid phase was removed by centrifugation, and the absorption of the supernatant liquids was measured at 281 mμ in a Beckman spectrophotometer as a measure of their protein content. Analysis of the curve shown in Fig. 5 indicates, according to the discussion of Herriott, the presence of 95 to 97 per cent of one protein component.

The ultraviolet absorption spectrum of a twice crystallized preparation is shown in Fig. 6. It exhibits the usual maxima, owing to tyrosine, and tryptophan.

Osmotic Pressure, Molecular Weight, and Turnover Number—The molecular weight of four times crystallized protein was calculated from osmotic pressure measurements made by the procedure of Bull (15). The enzyme was dissolved in 0.2 M acetate buffer, pH 6.1, made up with water saturated with calcium sulfate. The protein was dialyzed against the solvent for 1 week at 5°. Equilibrium in the osmometers was reached from both sides in 5 days at 30°. The final values were 2.86 and 2.72 cm. of water for the osmotic pressure of solutions containing respectively 0.656 and 0.643 gm. of protein per 100 gm. of solvent. The molecular weight was therefore calculated as 59,500 ± 900.

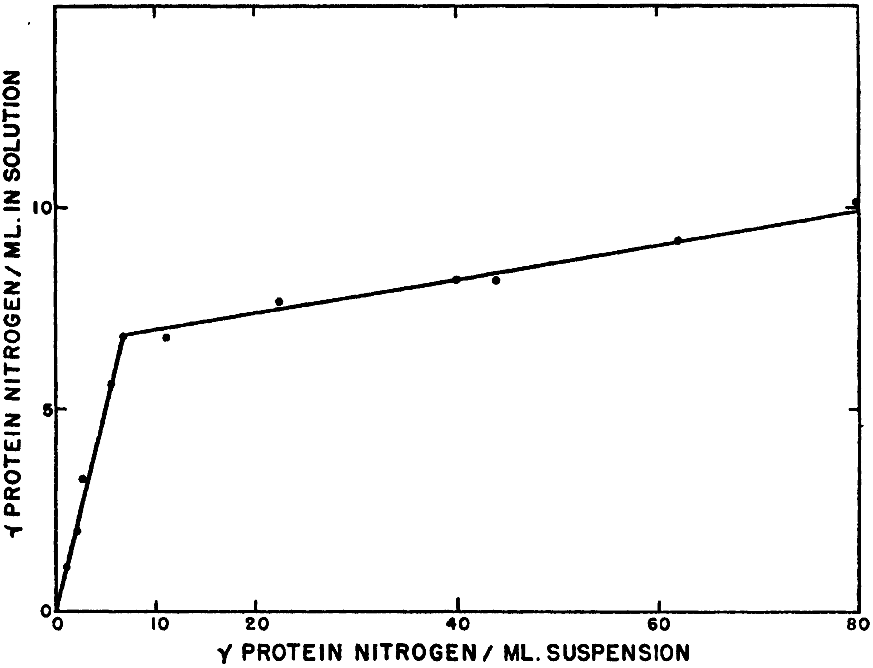

FIG. 5. Solubility of three times crystallized malt α-amylase in half saturated ammonium sulfate at pH 5.95 in the presence of increasing quantities of solid phase.

Calculation of the turnover number from this value and the data of Fig. 7 indicates 19,000 bonds hydrolyzed per minute per molecule of enzyme (molecular weight taken as 59,500) when the total number of glycosidic bonds per molecule of enzyme in the digestion mixture was 4 × 10^6. The turnover number for crystalline pancreatic amylase, calculated from the data of Meyer, Fischer, and Bernfeld (16) and by Fischer and Bernfeld (17), is 25,000, with 45,000 as the molecular weight when 4×10^4 glycosidic bonds were present per molecule of enzyme.

Enzymic Action—The elimination of β-amylase in the early stages may be justly assumed, since this enzyme in malt is known to be relatively sensitive to heat. Furthermore, the rate of production of reducing substances from starch (Fig. 7) is not in accord with the effect of even a small amount of the β enzyme. Thus the addition of calcium sequestering agents (0.01 M "tetraphosphate" or 0.01 M hexametaphosphate, pH 5.0) was found to have no effect upon β-amylase, but destroyed *all* the amylolytic activity of the α preparations.

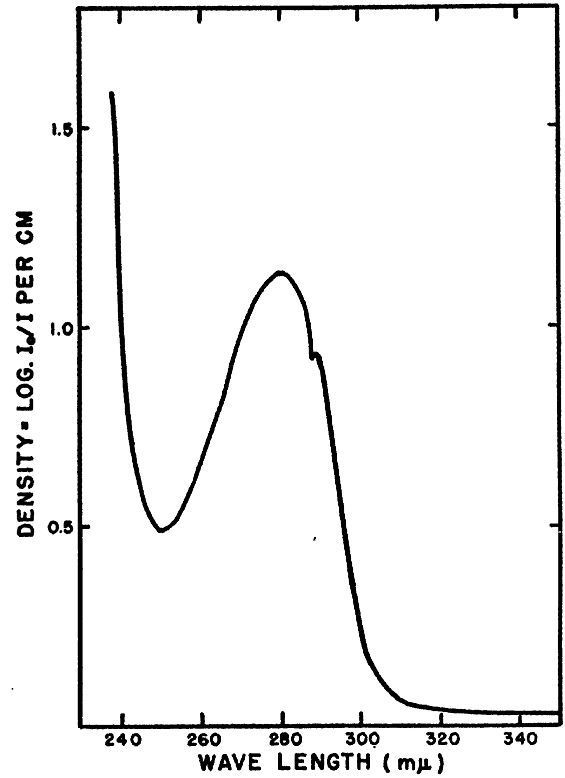

Fig. 6. Ultraviolet absorption of crystalline malt α-amylase (0.0083 mg. of protein nitrogen per ml. in 0.1 M acetate, pH 6.0, containing $CaSO_4$).

The extent of digestion of soluble starch solutions by two different concentrations of crystalline amylase is shown in Fig. 7. Formation of reducing substances appears to follow the same kinetics as observed by Bernfeld and Studer-Pécha (18) with partially purified malt α-amylase acting on amylose. This behavior has been regarded as characteristic of α-amylase action. On the other hand, Fig. 1 indicates that the reducing value at the "achromic point" (no color of the digest with iodine) was 24 per cent compared to the value of 30 per cent reported by Hanes and Cattle (19). However, this difference may be due to the differences in the iodide and iodine concentrations used to obtain the color. Swanson (20) has shown that the absorption maxima of the dextrin-iodine complexes shift towards smaller wave-lengths during digestion of amylose by α-amylase.

Whereas partially purified preparations of malt α-amylase rapidly lost all activity when dialyzed against distilled water, it was difficult to destroy the activity of a solution of crystals completely by the most thorough dialysis (20 ml. was dialyzed for 1 week against a total of 30 liters of distilled water). Such dialyzed enzyme lost 84 per cent of the activity and 59 per cent of the protein became insoluble. This preparation still gave a positive flame test for calcium. Furthermore, crystals which were washed thoroughly against 0.4 saturated ammonium sulfate containing no calcium still contained 0.13 per cent of calcium. In this preparation the molar ratio of protein of molecular weight 60,000 to calcium is 2. It would seem that crystalline preparations still require calcium for activity, but that it is more difficult to remove it from the crystalline enzyme. Lyophilization of a solution of crystallized enzyme dissolved in saturated calcium sulfate water resulted in almost complete loss of activity.

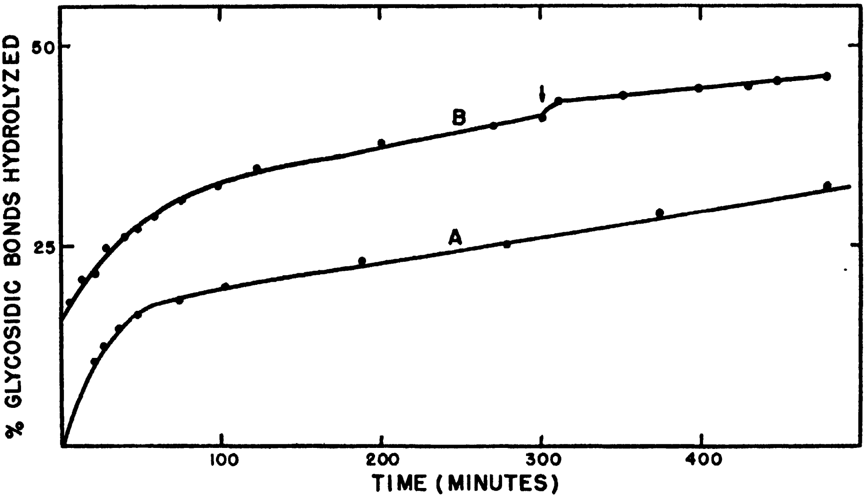

FIG. 7. Hydrolysis of soluble starch by crystalline malt α-amylase at 30°, pH 4.6. The concentration of starch was 12 mg. per ml. The concentration of enzyme protein was 0.7 γ per ml. for Curve A and 13 γ per ml. for Curve B. An additional amount of enzyme was added at 303 minutes to bring the total concentration of enzyme protein to 20 γ per ml.

According to Lane and Williams (21), inositol is an active constituent of pancreatic α-amylase. They found that the γ isomer of hexachlorocyclohexane acted as an inhibitor and that inositol reversed this inhibition. When crystalline malt α-amylase was dissolved in 50 per cent dioxane and 2 mg. per ml. of a mixture of isomers of hexachlorocyclohexane were added, no decrease in activity was observed after 24 hours at 5°. A control containing dioxane but no hexachlorocyclohexane also lost no activity under the same conditions. Qualitative tests for inositol as described by Salkowski (22) were negative.

SUMMARY

Isolation and crystallization of the α-amylase of germinated barley (malt) has been described in detail. The procedure consists essentially of heating concentrated malt extract, precipitating the remaining protein with ammonium sulfate, adsorption of the enzyme from an alcoholic solution on wheat starch granules, and crystallization of the eluted enzyme from ammonium sulfate. Behavior of the fractions leading to crystallization and some of the properties of the crystalline substance, including molecular weight, purity, and ultraviolet absorption spectrum, are reported. The kinetics of hydrolysis, the requirement of the enzyme for calcium, and the apparent absence of inositol in the enzyme are also discussed.

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THE EXTRACTION OF FOLIC (PTEROYLGLUTAMIC) ACID CONJUGATE FROM YEAST

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The conjugate of folic acid (pteroylglutamic acid, vitamin B_c) in yeast was isolated by Pfiffner *et al.* (1) and determined to be pteroylhexaglutamyl glutamate (2). Recently this group has published details of the concentration of the conjugate (3). The present paper describes a simple method for producing a concentration of the conjugate of from 40- to 100-fold by differential extraction of columns of yeast with alcohol-water mixtures. Further purification was attained by fractional precipitations in 70 per cent alcohol at different pH levels.

EXPERIMENTAL

Assay Methods—Samples were assayed for "potential folic acid" (4) with *Streptococcus lactis* (*faecalis*) R (5), and *Lactobacillus casei*, with use of a vitamin B_c standard.[1] The medium for the *L. casei* assay (6) was supplemented with a folic acid-free, lead-treated solution of yeast extract (7, 8). On the basis of the ratio of 1:2.8 between the molecular weights of pteroylglutamic acid and the conjugate (2), all assay values were expressed in terms of the conjugate, in which form the vitamin existed throughout these fractionations.

Conditions for Extraction of Conjugate from Yeast—A slurry was made of flaked brewers' yeast with 60 per cent alcohol (formula 3A, 90:10 ethanol-methanol was used throughout) and poured into a column. Continued percolation with 60 per cent alcohol removed most of the extractives but very little conjugate. Following this, the conjugate was extracted by 45 to 50 per cent alcohol. The extraction of conjugate was improved by adding to the 45 to 50 per cent alcohol either HCl to make 0.01 N or NaH_2PO_4 to make 0.1 M. The data in Table I show that this type of extraction produced excellent yields of conjugate and a high degree of purification. In Experiments 3 and 4, 75 to 80 per cent of the conjugate, with about 100-fold increase in concentration, was contained in just about the amount of extract (1.3 cc. per gm. of yeast) required for complete exchange of the liquid in the column. On a larger scale the yields and the extent of concentration were somewhat less, due to difficulty in duplicating the conditions for ideal percolation attainable with smaller columns.

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[1] Acknowledgment is made to Parke, Davis and Company and to the Lederle Laboratories Division, American Cyanamid Company, for vitamin B_c.

In extraction with phosphate solutions, the true degree of concentration was somewhat higher than indicated by determination of the total solids, due to phosphate in the extract. However, the amount of phosphate in the extracts was much less than might have been expected. Although the 45 per cent alcohol originally contained 0.012 gm. of NaH_2PO_4 per cc., calculations from volume and weight data show that *total solids* in the active extracts were only about one-half of this amount. Not until the conjugate was almost completely extracted did phosphate appear in the extract in the expected amounts. Therefore the extraction of conjugate was accompanied by adsorption of phosphate by the yeast.

In a larger scale experiment a 250 liter crock with a bottom side-outlet was fitted for percolation with a column support of crushed rock and sand and 45.4 kilos of flaked brewers' yeast were added as a slurry with 76 liters of 60 per cent alcohol. Percolation with 60 per cent alcohol was maintained until 380 liters of extract had been collected and no liquid remained above the yeast. At this point percolation with 50 per cent alcohol containing 0.01 N HCl was started and the next 38 liters of extract were combined with the first fraction (Fraction 1, Experiment 5, Table I). The extract was collected in three additional fractions. Fraction 2 (38 liters) contained very little conjugate, because the solvent was mostly 60 per cent alcohol which had remained in the column. Most of the conjugate was in Fraction 3 (200 liters), which contained 65 per cent of the activity of the yeast. More exhaustive extraction, as indicated by Fraction 4 (150 liters), did not significantly increase the yield of conjugate. Fractions 3 and 4 were combined[2] and concentrated *in vacuo* to 8 to 10 liters.

Further Concentration of Conjugate—In further experiments (Table II), advantage was taken of the fact that the conjugate is more soluble at pH 3 than at pH 6 in 70 per cent alcohol. Of the above extract, 7220 cc., which were derived from 40 kilos of yeast, contained 1464 mg. of conjugate in 1117 gm. of solids. To this solution were added, with stirring, 130 cc. of 10 N HCl to adjust the pH to 3.0 and anhydrous denatured alcohol to make a volume of 26 liters. The precipitate which was formed, after washing with 3 liters of 70 per cent alcohol and drying, weighed 610 gm. and was relatively inactive (Fraction 1, Experiment 1). The 70 per cent alcohol solution was adjusted to pH 6.0 with 102 cc. of 10 N NaOH. After decantation and washing with 70 per cent alcohol the dried active precipitate (Fraction 2) weighed 104.5 gm. and contained 1.156 gm. of conjugate, equivalent to about 50 per cent recovery of that in the yeast. When refractionated by a similar procedure, 10 gm. of the material yielded 3.95

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[2] Fraction 4 was combined with Fraction 3 before its low potency was known.

TABLE I

Extraction of Folic Acid Conjugate from Yeast by Percolation with Alcohol-Water Mixtures*

Experiment No.	Weight of yeast	Dimensions of column	Extractant		Extract			
			Alcohol	Additions to alcohol-water	Fraction No.	Volume	Total solids	Total conjugate
	gm.	*cm.*	*per cent*			*cc.*	*gm.*	*mg.*
1	100	3 × 30	60		1	1000	23.0	0.72
			50	0.01 N HCl	2	920		4.73
2	100	3 × 30	60		1	1000	23.2	0.76
			50	0.07 M NaH_2PO_4	2	910		4.90
3	200	3 × 60	60		1	2120	46.6	0.72
			60–45†		2	250	0.66	0.62
			45	0.1 M NaH_2PO_4	3	250	1.70	8.62
			45	0.1 " "	4	164	0.92	0.44
			45	0.1 " "	5	1340	21.6	0.72
4	400	3 × 120	60		1	4280	92.8	1.92
			60–45†		2	580	1.4	1.12
			45	0.1 M NaH_2PO_4	3	224	1.72	10.4
			45	0.1 " "	4	320	1.72	7.96
			45	0.1 " "	5	800	9.16	0.14
						liters		
5	45,400	53 × 38	60		1	418	9489.0	326.9
			60–50†		2	38	149.8	16.3
			50	0.01 N HCl	3	200	749.1	1679.8
			50	0.01 " "	4	150	408.6	84.0

* Flaked brewers' yeast containing 0.059 mg. of conjugate per gm.

† The fraction contains 60 per cent alcohol from the column.

TABLE II

Effect of pH in Fractionation of Folic Acid Conjugate in 70 Per Cent Alcohol

Experiment No.	Fraction No.	Description	Total solids	Total conjugate
			gm.	*mg.*
1		Concentrate from 40 kilos of yeast (Experiment 5, Table I)	1117	1464
	1	70% alcohol ppt., pH 3.0	610	207.5
	2	70% " " " 6.0	104.5	1156
	3	70% " filtrate, pH 6.0	520	62.7
2		Fraction 2, Experiment 1	10.0	110.6
	1	70% alcohol ppt., pH 3.0	1.3	7.6
	2	70% " " " 5.5	3.95	89.3
	3	70% " filtrate, pH 5.5	4.8	13.2

gm. of a precipitate which was about twice as potent and contained 2.2 per cent conjugate. This represents a concentration from yeast of nearly 400-fold with an over-all yield of 40 per cent.

SUMMARY

Folic acid conjugate was concentrated by percolating yeast in a column with alcohol-water mixtures. Extractives which contained little activity were largely removed with 60 per cent alcohol. The conjugate was then extracted with slightly acidic 45 to 50 per cent alcohol. Such extracts contained up to 6 mg. of conjugate per gm. of solids, or about 100 times the content in yeast. Further concentration was obtained by removal of inactive solids from 70 per cent alcoholic solution at pH 3, followed by precipitation of an active fraction at pH 5.5 to 6.0. By these methods a concentration of conjugate of 22 mg. per gm. of solids was reached.

Acknowledgment is made to Frances M. Phillips and Abraham J. Brook for technical assistance in this work.

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THE INFLUENCE OF DIET ON THE RIBOFLAVIN CONTENT AND THE ABILITY OF RAT LIVER SLICES TO DESTROY THE CARCINOGEN *N,N*-DIMETHYL-*p*-AMINOAZOBENZENE*

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The production of liver tumors in the rat by the oral administration of *N,N*-dimethyl-*p*-aminoazobenzene (DMB) is markedly influenced by diet. Our early work (1, 2) showed that the maintenance of normal hepatic riboflavin levels was of importance in protecting rats against tumor formation. The work of Griffin and Baumann (3) and Miller, Miller, Kline, and Rusch (4) has emphasized the relationship between hepatic riboflavin levels and the carcinogenic action of DMB and related compounds. Riboflavin supplements are not effective in maintaining hepatic riboflavin levels or preventing tumor formation in the absence of adequate protein intake (2).

In a study (5) from this laboratory of the effect of diet on the ability of rat liver slices to destroy DMB it was found that the maintenance of rats on a brown rice-carrot diet, which favors tumor production by DMB (6, 7), decreased this activity of rat liver. It was also observed that the addition of protective supplements such as dried brewers' yeast or riboflavin and casein prevented this decrease. Measurement of the riboflavin content of the livers of rats on these diets indicated that, as the riboflavin level fell, the ability to destroy DMB also decreased.

The use of brown rice diets in the preceding study (5), while suggesting that the changes observed were due to inadequate riboflavin and protein intake, made it necessary to rule out the presence of a "toxic" factor in the rice as the responsible agent. For this reason, and for the purpose of controlling the intake of other dietary constituents, the experiments have been repeated with diets which did not contain rice or carrot. As the Wisconsin group (8, 9) have published extensively on the effect of casein-dextrose diets on the carcinogenic action of DMB, the diets selected were modeled on the ones investigated by that group.

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† Fellow of the Committee on Growth of the National Research Council.

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Methods

The destruction of DMB by liver slices was measured as described in our earlier report (5) and depended on the recovery and spectrophotometric estimation of the DMB that was not destroyed. 100 γ of DMB were incubated with 150 mg. of liver slices in all experiments. Under the conditions of these experiments the destruction of DMB was proportional to the period of incubation. The recovery of added DMB at zero time was good, 90 to 95 per cent.

The basic diets used were of the casein-dextrose type described by Miller (9) for medium tumor incidence. All vitamin supplements, except those varied in the different experiments, were administered as specified (9). Halibut liver oil was omitted in view of the short time involved. In the 4 per cent protein diet the dextrose was increased to 87 per cent of the diet. The diets were fed *ad libitum.*

The rats used were of the Wistar strain, weighing between 100 and 150 gm. at the start of the experiment. Similar results have been obtained with Sherman strain rats obtained from the Rockland Farms (but are not included in this paper).

The riboflavin was measured by a modification of the fluorometric method of Hodson and Norris (10).

Results

The data obtained on hepatic riboflavin levels and the destruction of DMB by liver slices from rats maintained on various diets are shown in Table I. It can be seen that, as the riboflavin content of the diet was reduced, the riboflavin level in the liver and the *in vitro* destruction of DMB both decreased (Diets 1, 2, 3, and 4). In marked contrast, when thiamine (Diet 5) or choline (Diet 6) was omitted from the diet, there was no change in DMB destruction, and the hepatic riboflavin level was in the normal range, although in the absence of thiamine these values were slightly higher and in the absence of choline slightly lower than the controls. The failure of the omission from the diet of thiamine or choline to affect the ability of rat liver to destroy DMB thus supports the observation made when brown rice diets were used that a riboflavin-containing component of hepatic cells is either primarily or secondarily involved in the destruction of DMB.

The riboflavin level in the liver of rats is lowered also when the protein content of the diet is reduced (11, 12). As is shown in Table I (Diets 7 and 8), when the protein content of the diet used was reduced from 12 to 4 per cent, both the riboflavin level and the ability of liver to destroy DMB were decreased. Thus the protein level of the diet as well as its riboflavin content has been found to be of importance in maintaining hepatic riboflavin levels and high activity with respect to DMB destruction.

Biotin has been reported (13, 14) to increase tumor incidence when certain partially protective diets were used and adenine has been reported (15) to increase liver damage associated with DMB administration. The addition of adenine (Diet 9) and biotin (Diet 10) to the basal diet was without effect on riboflavin level or DMB destruction.

TABLE I

Riboflavin Content and Destruction of DMB by Liver Slices from Rats Fed Casein-Dextrose Base Diets for 14 to 20 Days

Diet No.		No. of rats	Riboflavin per gm. liver (wet weight)		DMB destruction by 150 mg. liver slices in 60 min.	
			Range	Average	Range	Average
			γ	γ	γ	γ
1	12% protein, 0.01 gm. vitamin B_2* per kilo	16	16.2–25.0	21.5	40–69	53.0
2	12% protein, 0.001 gm. vitamin B_2* per kilo	10	16.2–22.8	18.7	20–63	37.5
3	12% protein, 0.0005 gm. vitamin B_2* per kilo	11	12.3–18.8	16.0	20–29	25.7
4	12% protein, no added vitamin B_2	11	10.7–14.4	13.0	20–33	25.4
5	12% “ 0.01 gm. vitamin B_2 per kilo, no thiamine	10	21.8–25.2	22.8	47–75	58.9
6	12% protein, 0.01 gm. vitamin B_2 per kilo, no choline	11	13.3–25.0	19.7	45–68	57.5
7	4% protein, 0.01 gm. vitamin B_2 per kilo	9	11.0–21.8	16.5	20–49	34.7
8	4% protein, 0.001 gm. vitamin B_2 per kilo	10	9.2–19.4	14.0	19–40	31.5
9	12% protein, 0.01 gm. vitamin B_2 per kilo, 0.1% adenine	5	21.0–25.2	22.3	51–71	62.4
10	12% protein, 0.01 gm. vitamin B_2 per kilo, + 0.002 gm. biotin per kilo	6	19.2–23.2	20.8	48–55	51.7
11	12% protein, 0.01 gm. vitamin B_2 per kilo, 0.06% DMB	9	16.4–22.4	18.3	18–48	26.1
12	12% protein, 0.001 gm. vitamin B_2 per kilo, 0.06% DMB	10	11.0–14.8	12.2	12–26	18.4

* Riboflavin.

The relationship between the riboflavin level in the liver and the ability to destroy DMB is shown more clearly in Fig. 1. Rats fed Diets 1 through 8, Table I, have been plotted. The data show that the decrease in the ability to destroy DMB is most marked as the riboflavin content falls from 22 to 15 γ per gm. of liver tissue. Rats maintained on a ration of dog chow usually have between 25 and 30 γ of riboflavin per gm. of liver tissue but the destruction of DMB is not much greater than for livers containing 22 to 25 γ per gm. Conversely it has been found that rats maintained on a brown rice-carrot diet for periods of 80 to 100 days containing 8 to 10 γ of riboflavin per gm. of liver destroyed only slightly less DMB than the livers containing 12 to 15 γ per gm. Thus the striking drop in the ability to destroy DMB occurs to a large extent as the riboflavin content is decreased from 22 to 15 γ per gm. and the curve flattens out at both high and low riboflavin levels.

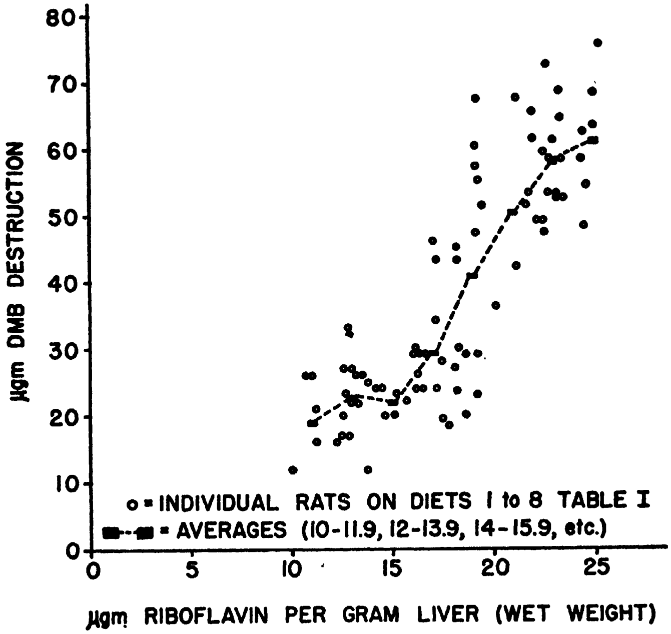

FIG. 1. Relationship between the riboflavin content and destruction of DMB by liver slices from rats fed casein-dextrose base diets for 14 to 20 days. The solid points connected by the dotted line represent the average values for each 2 γ range of riboflavin level; *i.e.*, the point at 15 γ per gm. includes all animals containing from 14.0 to 15.9 γ per gm. of liver.

The results presented in the preceding paragraphs were obtained without the inclusion of the carcinogen DMB in the diet. The inclusion of DMB in diets of the brown rice type was shown to depress the riboflavin level more than the diet alone (1). This observation has been extended to other diets and azo compounds by Griffin and Baumann (3). As is shown in Table I, the inclusion of 0.06 per cent DMB in the 0.001 and 0.0001 per cent riboflavin diets (Diets 11 and 12) decreases both the riboflavin level and the ability of slices of the liver to destroy DMB, more than the diet alone.

DISCUSSION

The data presented extend the observation (5) originally made on rats maintained on brown rice diets that the rate of destruction of the hepatocarcinogen DMB by rat liver slices is related to their riboflavin content. Although the metabolism of DMB *in vivo* is not completely worked out, it involves a complex series of reactions leading to cleavage of the azo linkage (16), demethylation of the amino group (16, 17), oxidation of the *p'* position (16, 17), the accumulation of an unknown but colored derivative in the liver (18), as well as the excretion of the metabolites in conjugated form (16, 17). As neither the end-products nor the intermediary products of the destruction of DMB by liver slices have been identified, it is difficult to assess the significance of the apparent correlation between riboflavin content and DMB destruction. However, it appears likely that the azo linkage is the grouping attacked in the slice experiments, as the destruction of *p*-aminoazobenzene is also decreased by diets lowering the riboflavin content of the liver (5).

Mueller and Miller (18) have recently reported that diphosphopyridine nucleotide (DPN) is involved in the destruction of DMB in liver homogenates. This has also been observed in our laboratory. Measurement of DPN content (1, 19) of the livers of rats fed high and low tumor incidence diets of the brown rice type showed that the DPN level was depressed by the administration of DMB in the diet but not by the diet alone, in contrast to the variation of riboflavin levels as a function of these diets. Thus it would appear that DPN is not a limiting factor in the liver slice experiments reported in this paper.

SUMMARY

1. The limitation of the riboflavin or protein intake but not the thiamine or choline intake has been found to reduce both the riboflavin concentration in rat liver and the ability of rat liver slices to destroy the liver carcinogen *N*,*N*-dimethyl-*p*-aminoazobenzene.
2. The addition of biotin or adenine to the diet was without effect on either the riboflavin level or the destruction of the carcinogen by liver slices.
3. Data showing the relationship between the riboflavin level in the liver and the ability to destroy the carcinogen are presented.
4. The inclusion of the carcinogen in the diet further reduced the hepatic riboflavin level and the ability of liver slices to destroy it.

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MEASUREMENT OF OXYGEN UPTAKE UNDER CONTROLLED PRESSURES OF CARBON DIOXIDE

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(Received for publication, February 21, 1949)

Aerobic experiments with the Warburg technique are carried out essentially in the absence of CO_2 because alkali in the center well removes CO_2 as it is produced (1, 2). Various methods have been devised to correct for this shortcoming, but these methods are cumbersome, requiring two flasks or special apparatus (3). This paper presents a method of measuring oxygen uptake at a constant CO_2 pressure. A suitable solution in the center well maintains a chosen concentration of CO_2 in the flask analogous to the manner in which a buffer holds pH constant. The solution contains diethanolamine (2,2′-iminodiethanol), HCl, and $KHCO_3$ in amounts that are varied to give the desired CO_2 concentration. The solutions equilibrate sufficiently rapidly and have a large enough capacity under the usual experimental conditions in a Warburg flask of the conventional type, under CO_2 up to 3 volumes per cent of the gas. Robbie (4) has used a similar principle to maintain constant HCN in the Warburg flask. Warburg (5) was the first to apply a modification of this principle (see "Discussion").

EXPERIMENTAL

Methods

All experiments were performed at 38° with a Warburg apparatus shaken at 230 strokes per minute of 2.5 cm. length. The flasks were conical, of 12 to 16 ml. volume, containing 3.0 ml. of liquid in the main part of the flask, and with center wells 15 mm. high by 8 mm. inside diameter, containing 0.6 ml. of solution unless otherwise noted. The lip of the center well was greased to prevent creeping, and two pieces of Whatman No. 42 filter paper, each 22 by 40 mm., one rolled into a cylinder and the other folded in accordion fashion (4), were placed in the center well in such a manner that they projected about 7 mm. The folded paper was used alone in one experiment and the results indicated it to be equally satisfactory below about 1 per cent CO_2.

Center Well Solutions—Technical grade (Eastman) diethanolamine was used after it was diluted to 60 per cent by volume with water and warmed with a little charcoal to remove most of the color and odor. The per cent CO_2 in equilibrium with solutions containing 10 ml. of 60 per cent diethanolamine, various amounts of 6 N HCl, water to make 15 ml., and 3.0 gm. of powdered $KHCO_3$ is given in Fig. 1. Less $KHCO_3$ is used to obtain lower pressures. The $KHCO_3$ dissolves in 1 to 2 hours at room temperature with occasional shaking. Solutions were allowed to stand at least overnight before use. They are stable and solution enough for many experiments can be made at one time if kept in stoppered bottles. Experiments by Mr. Erich Hirschberg showed that at 19° and 27° the equilibrium pressures were one-fourth and one-half as great, respectively, as at 38°. Perhaps triethanolamine would be superior at these temperatures.

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* Merck Postdoctoral Fellow in the Natural Sciences under the National Research Council.

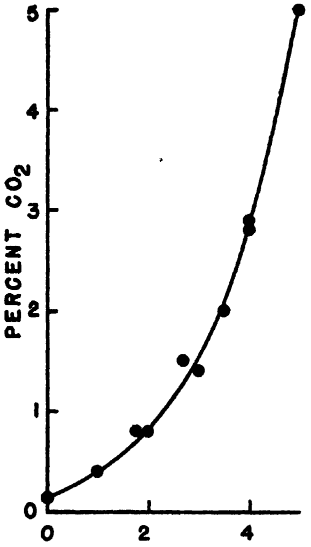

FIG. 1

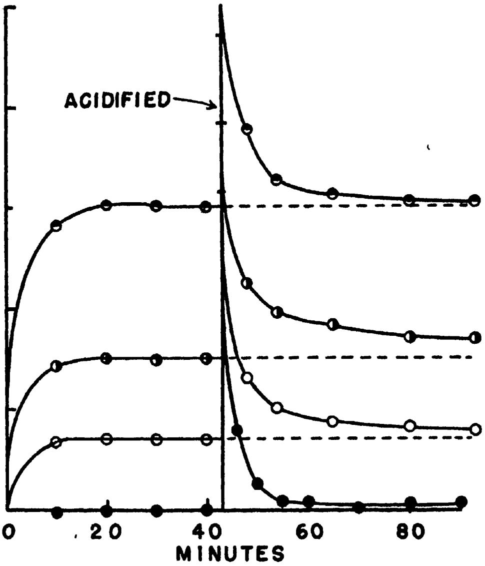

FIG. 2

FIG. 1. Dependence of CO_2 on composition of center well solution. For a description, see the text.

FIG. 2. Rate of uptake of released CO_2. 320 μl. of CO_2 were released at 43 minutes. ●, NaOH; ○, 0.7 per cent CO_2; ◑, 1.5 per cent CO_2; ⊖, 3 per cent CO_2 (Dickens-Simer flasks). The points are averages of duplicate experiments.

Determination of CO_2 Concentration—To determine the equilibrium concentration of CO_2, a solution in the center well was shaken on the Warburg apparatus with a solution containing a drop of 0.1 per cent phenol red plus a concentration of K_2CO_3 such that the pH at equilibrium lay in the range of the indicator (6). The per cent CO_2 in the gas was obtained from the pH estimated visually and the bicarbonate concentration (1 HCO_3^- ion for each K^+ ion or 2 times the concentration of K_2CO_3) by means of the equation (3), per cent $CO_2 = 6.2 \times 10^9 (H^+)(HCO_3^-)$. This procedure was found to be in error by less than 20 per cent, or 0.1 pH unit, when checked by measuring the increase in pressure when excess HCl was dumped from the side arm into a K_2CO_3 solution and then comparing with the per cent CO_2 calculated from the pH of an indicator-carbonate solution in the center well. 1 per cent CO_2 is equal to 100 mm. of manometer fluid (Brodie's solution).

Rate and Final Equilibrium—In the experiments shown in Fig. 2, flasks containing 2.7 ml. of 5×10^{-3} M $KHCO_3$, 0.3 ml. of 0.5 N HCl in the side arm, and 0.6 ml. of diethanolamine solution in the center well were shaken for 43 minutes; then the acid was tipped into the $KHCO_3$, releasing CO_2 (7). The CO_2 concentrations at equilibrium were measured in separate flasks.

For comparison, the diethanolamine solution was replaced by 0.2 ml. of 2 N NaOH plus a piece of 15 by 25 mm. filter paper. This set-up may not be optimum, but is the one used in this laboratory. Since CO_2 will diffuse into the alkali from bicarbonate, 0.1 ml. of 0.5 N NaOH was added to the bicarbonate, and the acid was dumped at 13 minutes; however, the data are plotted with the others for better comparison.

Flasks with empty center wells were used to measure the amount of CO_2 released. In these flasks, also, NaOH was added to the bicarbonate. The data obtained are not shown; the CO_2 pressure had risen to the equilibrium level of about 300 mm. before the next reading was made.

Initial equilibrium was not attained in the first 10 minutes at the higher concentrations, but this can be obviated by exhaling through a pipette into each flask a moment before it is put on, so that CO_2 will be present in the gas phase initially. The rates of return to equilibrium were similar to the rate of absorption by NaOH. A better test is given by the homogenate experiments described below. Final equilibrium was attained within the error to be expected of Warburg measurements. As the pressure of CO_2 or the total amount absorbed is increased, the error will increase, so that 1.5 per cent CO_2 seems to be an upper limit for this method, though an arbitrary correction can be made if the rate of uptake is adequate.

It is probable that only a small part of the solution in the center well comes to equilibrium with the gas phase, as shown by the superior equilibration by Dickens-Simer flasks (3), even at 3 per cent CO_2, with 1 ml. of solution and three glass or metal beads in the large center well.

Comparison by Means of Homogenate System—Fig. 3 is a plot of rate of oxygen uptake (calculated as if CO_2 were absent) against time when essentially the oxalacetic oxidase system (8), to which approximately equilibrium amounts of KHCO$_3$ were added, was used. Two solutions in the center well are compared with NaOH and with nothing in the center well; the difference between the two determinations is a measure of the CO$_2$ which the solution in the center well must absorb. This volume appears to be 75 per cent of the oxygen uptake. The uptake in the presence of CO$_2$ was the same as in its absence, showing that the evolved CO$_2$ was efficiently absorbed, though possibly CO$_2$ stimulates the reaction enough to compensate for incomplete CO$_2$ uptake. In Fig. 4, maximum rates for this system with various amounts of homogenate are compared at 0 and 1.5 per cent CO$_2$. The rates lie together within experimental error up to at least 70 µl. per 10 minutes, showing

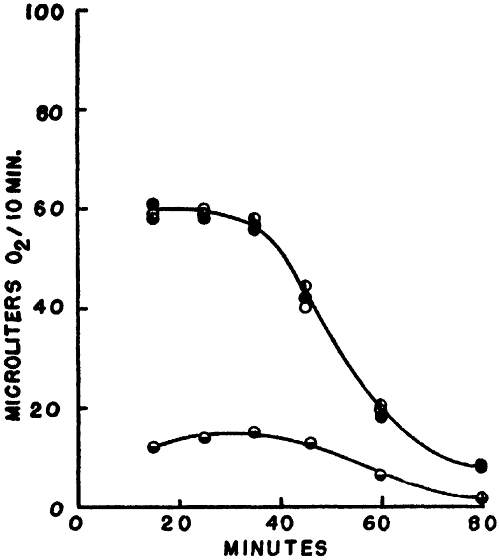

FIG. 3

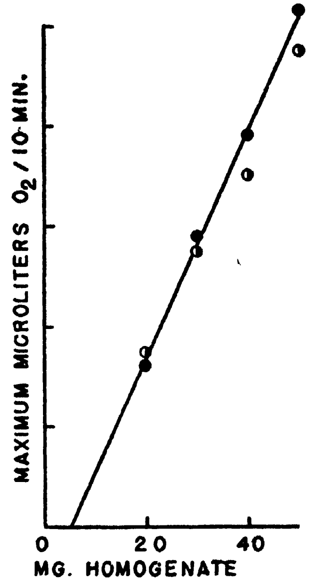

FIG. 4

FIG. 3. Oxygen uptake at 0, 0.5, and 1.0 per cent CO$_2$. The flasks contained (final concentrations) 0.0067 M K phosphate, 0.067 M KCl, 1.3 × 10^{-5} M cytochrome *c*, 0.0033 M MgCl$_2$, 0.001 M K adenosine triphosphate, 0.0018 M K pyruvate, 0.0018 M K oxalacetate, variable KHCO$_3$, and 0.3 ml. of 10 per cent rat kidney homogenate in isotonic KCl, in a volume of 3.0 ml. at initial pH 7.1. The points are averages of duplicate experiments. ●, no KHCO$_3$, NaOH in center well; ○, 10^{-3} M KHCO$_3$, 0.5 per cent CO$_2$; ◐, 2 × 10^{-3} M KHCO$_3$, 1.0 per cent CO$_2$; ⊖, no KHCO$_3$, nothing in center well.

FIG. 4. Maximum rates of oxygen uptake at zero and 1.5 per cent CO$_2$. Conditions as in Fig. 3 with variable amounts of homogenate. The points are averages of duplicate experiments. Flasks with 1.5 per cent CO$_2$ contained 2 × 10^{-3} M KHCO$_3$. ●, NaOH; ◑, 1.5 per cent CO$_2$.

that the mixture in the center well absorbed 50 µl. per 10 minutes of CO$_2$ efficiently.

Higher CO$_2$—If CO$_2$ above 1.5 per cent is desired, it is necessary to start with CO$_2$ in the gas phase to eliminate the otherwise long equilibration period. This CO$_2$ can be introduced either by gassing by a conventional method (3), or by exhaling into each flask through a pipette until the CO$_2$ content has a composition similar to that of the breath. Fig. 5 presents a comparison of rate of oxygen uptake with NaOH in the center cup and center well mixtures giving 2 per cent and 3 per cent CO$_2$. In the flasks not gassed the initial equilibration took about an hour; exhaling into the flask or gassing (same results) reduced this time to 30 minutes at 3 per cent CO$_2$. The contents of the Dickens-Simer flasks were equilibrated in 20 minutes after gassing. The flasks containing 2 per cent CO$_2$ were in close agreement with those containing NaOH throughout the experiment. An experiment with 5 per cent CO$_2$ was totally unsuccessful.

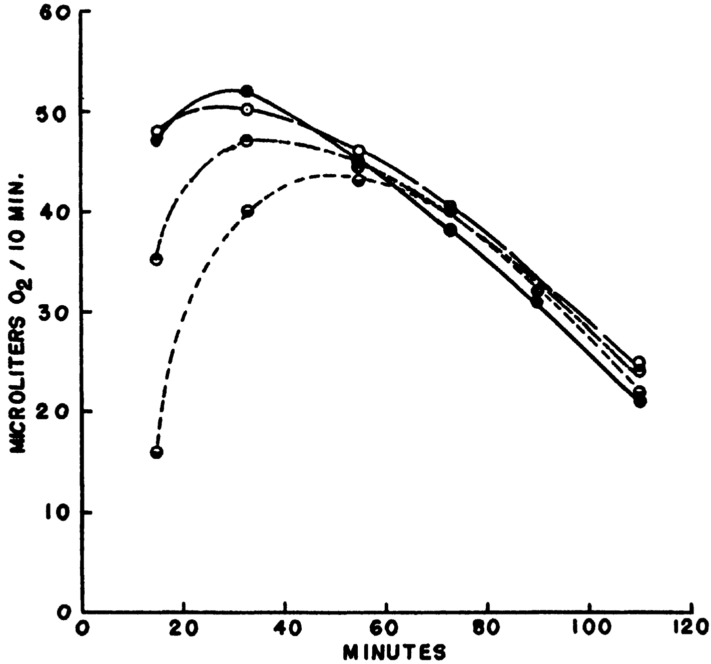

Fig. 5. Oxygen uptake at 2 and 3 per cent CO$_2$. Effect of gassing. The conditions are as in Fig. 3 with 0.25 ml. of 10 per cent rat kidney homogenate in isotonic KCl per flask. ●, NaOH; ○, 2 per cent CO$_2$, 4×10^{-3} M KHCO$_3$ in solution, CO$_2$ initially introduced into flasks; ⊖, 3 per cent CO$_2$, 6×10^{-3} M KHCO$_3$; ◓, 3 per cent CO$_2$, 6×10^{-3} M KHCO$_3$ in solution, CO$_2$ initially introduced into flasks. The points are averages of duplicate determinations.

DISCUSSION

The level of CO_2 usually used is 5 per cent (3), 3 times the concentration attainable by the technique described in this paper, but this limitation has not been shown to be significant for any experiment (1, 2). By improving the design of the center well, giving it a greater area and better mixing, about 3 per cent CO_2 can probably be used, when there is a preliminary gassing of the flask. A center well presenting a greater area to the gas phase is better because the rate of absorption is proportional to the area (9). It is advantageous to have as large a rate of absorption as possible, so that initial equilibrium is set up rapidly. Dixon and Elliott (7) showed that the CO_2 pressure in the flask must be higher than the equilibrium pressure over the center well (taken as zero in their case) by an amount equal to the rate of CO_2 output divided by the constant of rate of absorption. This excess pressure can be calculated (from Fig. 2) to be 12 to 17 mm. of manometer fluid at a rate of CO_2 output of 50 μl. per 10 minutes. As the rate of reaction decreases with time, the CO_2 pressure drops and this is erroneously measured as oxygen uptake. This error, like the error of opposite sign which results from increased equilibrium pressure due to change in composition of the center well solution, appears gradually during the reaction. It is not large under the conditions described in this paper and can be decreased by increasing the rate of absorption. Another advantage of a large center well is that it can hold a greater effective volume of solution, which leads to smaller changes of composition on absorbing a given amount of CO_2, and hence a smaller change in equilibrium pressure.

Diethanolamine is an inexpensive, non-toxic material used industrially for removing CO_2 from gas mixtures. It appears from its dissociation curves to be an excellent material for obtaining concentrations of several per cent CO_2. The reactions probably involve carbamate formation, go in two or more steps, and require two amines per CO_2 molecule. Viscosity at higher concentrations decreases the rate of reaction, which is optimum at 3 M under certain conditions (10, 11).

For pressures above 3 per cent, diethanolamine will probably have to be replaced by another compound because its capacity and, more important, its rate of reaction with CO_2 decreases with increasing equilibrium pressures (10, 11). Other solutions were tried in the center well, and all were found to be inferior, including bicarbonate and KOH with borate plus bromine or carbonic anhydrase as catalysts (12), glycine which reacts rapidly to form a carbamate (13), glycine plus borate, triethanolamine (10), nembutal, and carbonate.

Warburg (5) studied O_2 pressure changes during photosynthesis by algae under constant CO_2, with carbonate-bicarbonate solutions in which the algae were suspended. The CO_2 pressure was quite constant under his conditions, but as discussed by Warburg, the method has limitations. The pH of the solutions in which the reactive material is suspended is determined by the CO_2 pressure, sometimes in an undesirable range. The volume of solution must be large in order for the salt concentration to be in a physiological range and yet buffer satisfactorily, and the CO_2 pressure must be low; for instance, the highest concentration of CO_2 used was 0.27 per cent obtained with 10 ml. of solution at pH 9.2 containing 0.015 M Na_2CO_3 and 0.085 M $NaHCO_3$. The rate of equilibration may not be adequate in a system which gives off CO_2.

We have not searched the literature for systems in which CO_2 is important. Warren (1) and Laser (2) list a number of references indicating that CO_2 aids the functioning of tissue preparations. Certain microorganisms require CO_2, and this technique (14) might be used in studies on photosynthesis and fixation of CO_2 in plants and animals. An advantage of the technique is that the pH is held more constant, which is useful when low buffer concentrations are used. The method is no more difficult to use than the conventional one with alkali and one shortcoming of the aerobic Warburg technique is avoided.

SUMMARY

A method is described for measuring oxygen uptake in the presence of a constant CO_2 concentration up to 3 per cent. Aqueous solutions of diethanolamine plus HCl and $KHCO_3$ in the center well of the Warburg flask act as a "buffer" to hold the CO_2 constant. The method was tested by measuring CO_2 released by acid and by a homogenate system.

I wish to thank Dr. Van R. Potter and Mr. Erich Hirschberg for their interest in the problem and for several valuable suggestions.

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INVESTIGATIONS ON THE NUTRITION OF LACTOBACILLUS LACTIS DORNER*

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Shorb recently reported an unidentified growth factor, LLD, which is required by *Lactobacillus lactis* Dorner. A liver extract preparation was established as an arbitrary standard and was assigned a potency value of 1000 LLD units per mg. (1). With the aid of a microbiological assay, with use of *L. lactis*, a crystalline material was isolated from liver extract and named vitamin B_{12} (2, 3). The LLD growth factor activity in certain complex materials is accounted for, at least in part, by the vitamin B_{12} content of these materials. Pure vitamin B_{12} has activity as a growth-promoting factor for *L. lactis* equivalent to 11,000 LLD units per microgram. Vitamin B_{12} also is active in the treatment of pernicious anemia and has growth-promoting activity for chicks fed diets deficient in animal proteins (4, 5).

Efforts to utilize *L. lactis* Dorner for assay of the LLD growth factor in this laboratory have met with some difficulty. Considerable variation was found in the response of *L. lactis* Dorner to standard solutions of crystalline vitamin B_{12} or concentrates rich in the LLD growth factor. Heavy growth frequently occurred in control tubes which contained no added LLD factor. Preliminary experiments on size of inoculum, pH of medium, temperature, length of incubation, and other known variants indicated that basic environmental factors were not controlled and may be responsible for the variations in response. Experiments showed that the relative oxygen and carbon dioxide tension of the environment profoundly affected the growth of this organism and its requirement for the LLD growth factor, supplied either by liver concentrate or by crystalline vitamin B_{12}.

A requirement for CO_2 and its utilization by many microorganisms has been established (6). However, the effect on microorganisms of varying O_2 tension, the oxidation-reduction potential of the medium, and the relationship of oxidizing and reducing agents to these factors are subjects of considerable controversy.

Ever since the Pasteur effect was demonstrated (7), investigators have attempted to define the mechanism of the inhibition of metabolic activities of anaerobes by oxygen. McLeod and Gordon (8) suggested that the inhibition noted was caused by the toxic effect of peroxides, which accumulated under aerobic conditions in the absence of catalase. This view was substantiated by Avery and Morgan (9), who demonstrated that peroxide was formed in aerobic cultures of *Pneumococcus* and showed that the addition of plant juices to such cultures destroyed the peroxide and resulted in good growth of their cultures. They also concluded that the production of catalase was the natural protective device of aerobic organisms which anaerobes lacked. This conclusion has since been contested by many workers who found that anaerobic organisms will grow in contact with air in the presence of aerobic cultures, even those known to be catalase-negative, or in the presence of respiring potato (10), or following the addition of reducing agents to the medium (11, 12). Further exception to McLeod's theory has been presented by Sherman (13), who reported that some obligate anaerobes do produce catalase. The inability of catalase to stimulate many anaerobes (14) is a fact which McLeod has noted himself (8).

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At present it is not clear whether the phenomena identified by these workers are applicable to a particular organism or whether the data found in the literature can be correlated into a comprehensive understanding of basic factors which govern the metabolism of aerobes and anaerobes. Since the lactobacilli are microaerophilic and demonstrate an adaptable mechanism which would lend itself to wide investigation of these factors, it is rather surprising that they have not been utilized more frequently for such investigation.[1]

In view of preliminary results relating growth of *L. lactis* Dorner to O_2 and CO_2 tension, it was hoped that investigations into the effects of varied concentration of these gases and related factors might yield interesting data on the limiting conditions for the growth of the organism.

EXPERIMENTAL

Medium—An amino acid basal medium, prepared according to Stokes *et al.* (15), was supplemented with 2 mg. per ml. of H_2SO_4 casein hydrolysate and 0.4 mg. per ml. of tomato juice eluate or its equivalent[2] per double strength medium.

Inoculum—*L. lactis* Dorner, strain 6a,[3] was carried in stabs containing 1 per cent dextrose, 1 per cent yeast extract, 0.5 per cent Salts A-B,[4] and 1.5 per cent agar, supplemented with 0.2 mg. per ml. of tomato juice eluate or its equivalent. A 20 to 24 hour transfer from a stab to liquid medium, similar to the above except for the absence of agar, was used for the inoculum. The broth culture was washed twice with sterile distilled water and resuspended in 10 ml. of sterile distilled water. 1 ml. of this suspension was diluted further in sterile distilled water to give a reading of 95 on the galvanometer of the Evelyn photoelectric colorimeter fitted with a 520 mμ filter. Stab transfers were made from the broth every 2 weeks and were stored in the cold room. It was found that stabs kept in the cold room more than 2 weeks occasionally failed to give good growth on transfer.

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[1] Callow did use *Streptococcus acidi lactici* (*Streptococcus lactis*) for some of her work (14).

[2] Tomato juice eluate preparations were kindly supplied by Dr. T. R. Wood. These preparations varied somewhat in TJ factor potency and LLD factor content. A particular preparation was useful only if the concentration required to give maximum growth in the presence of LLD factor did not itself stimulate a titer of more than 2 ml. in the absence of LLD factor.

[3] This organism was obtained from Dr. Shorb.

Procedure—Unless otherwise indicated, the experiments were conducted in Pyrex test-tubes 22 mm. × 180 mm. (these will be referred to as assay tubes), containing a total volume of 10 ml., obtained by diluting the sample being tested in 5 ml. of water and adding 5 ml. of double strength medium. Assay tubes were inoculated with 1 drop of the standardized suspension of *L. lactis* Dorner. Although other procedures were adapted to the nature of the experiments and will be described in other sections, assay tube controls, incubated under normal oxygen tension, were included in all experiments. All cultures were incubated at 37°. Readings were made after 3 days incubation and represent the number of ml. of approximately 0.1 N NaOH required to neutralize 10 ml. of the incubated medium. Averages from duplicate cultures are reported.

Throughout this paper the term LLD factor refers to the specific growth-promoting properties of liver concentrate or crystalline vitamin B_{12} for *L. lactis*, when grown under the above conditions.

Experiments on Effect of Environmental Gas Tension

In these experiments, the standard procedure, outlined above, was varied in the following manner. Shallow layer cultures (10 ml. in 125 ml. flasks) were incubated in 8 liter desiccators in the presence and in the absence of CO_2. The desiccators were evacuated and flushed three times with the desired gas and then a measured volume of the gas was introduced by displacement of water from a 10 liter reservoir bottle; so that the desiccator was filled just short of atmospheric pressure, to allow for expansion in the incubator and to insure against a break in the desiccator seal. Alkali was used to absorb CO_2 present as a contaminant in the gas or produced by *L. lactis*. The LLD growth factor was supplied by a liver extract, found to contain 100 LLD units per mg. (1).

The results shown in Table I indicate that *L. lactis* cannot grow in the absence of CO_2, even when the LLD growth factor is supplied. Maximum growth of this organism occurred in 100 per cent CO_2, even when liver extract, as a source of LLD growth factor, was absent from the medium. Lower concentrations of CO_2 mixed with nitrogen, producing anaerobic conditions (5 per cent CO_2 in this experiment, 2.5 and 1 per cent in other experiments), likewise permitted maximum growth of the organism in basal medium. However, growth failed to take place in the absence of the LLD growth factor, even under high CO_2 tension, when air was present. It was therefore concluded that, although CO_2 is essential to the growth of the organism, it is not related to the LLD growth factor requirement. The presence or absence of air does affect the requirement for this factor.

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[4] Salts A, stock solution, 25 gm. of K_2HPO_4, 25 gm. of KH_2PO_4, water to 250 ml.; Salts B, stock solution, 10 gm. of $MgSO_4 \cdot 7H_2O$, 0.5 gm. of NaCl, 0.5 gm. of $FeSO_4 \cdot 7H_2O$, 0.5 gm. of $MnSO_4 \cdot 4H_2O$, 0.5 ml. of 12 N HCl, water to 250 ml.

To investigate further the relationship between the LLD growth factor requirement of *L. lactis* and the degree of aeration, the response of the organism to given concentrations of the LLD factor in cultures of varying depth was determined. The LLD factor was supplied by liver extract and by vitamin B_{12} in separate experiments. Fig. 1 demonstrates that increased aeration decreases the growth response of the organism to a given concentration of vitamin B_{12}. There is an inverse relationship between the amount of LLD growth factor necessary to allow half maximum growth of *L. lactis* and the depth of the culture.

TABLE I

Effect of CO_2 on Growth of L. lactis

The values are acid (in 10^{-4} gm. molecule) produced in 10 ml. in 3 days.

Nature of medium		Air	Air + 5 per cent CO_2	95 per cent N + 5 per cent CO_2	Nitrogen + NaOH	Air + CO_2 from 50 gm. Na_2CO_3	100 per cent CO_2
Basal	Shallow layer culture	1.2	1.4	10.6	0.9	1.7	10.2
	Assay tube	1.6					
Basal + LLD*	Shallow layer culture	2.9	3.0	10.6	0.9	10.3	10.4
	Assay tube	10.6					

* The LLD growth factor content of the medium = 0.01 mg. per ml. of a liver extract which contained 100 LLD units per mg.

Oxidation-Reduction Potentials

An attempt was made to define the antagonism between the inhibitory effect of aeration and the growth-promoting activity of the LLD growth factor, supplied as crystalline vitamin B_{12}, in terms of oxidation-reduction potentials. The oxidation-reduction potentials of cultures were determined under conditions of varying oxygen tension in the presence and absence of the LLD growth factor. Platinum wire and saturated calomel electrodes were inserted directly into the solution or connected with an agar salt bridge and the observed potential recorded in millivolts. The platinum wire was rinsed in aqua regia, washed with distilled water, and heated to red heat before use. Electrodes were tested in buffered quinhydrone solution (pH 4.5 to 4.6) before and after each experiment.

Preliminary measurements on uninoculated medium demonstrated that standardization of procedure was difficult and many variables inherent in these measurements were not controlled. The system was poorly poised and freshly cleaned electrodes, which gave identical readings in quinhydrone solution, gave substantially different readings in culture medium. "Inertia" of electrodes, probably due to polarization, was frequently noted after continuous immersion for several hours. Measurements on inoculated cultures revealed additional sources of variation. A layer containing limited cell numbers, near the surface of deep stationary cultures, resulted in non-uniform potential conditions at various depths. In shaken or continuously bubbled cultures, air bubbles caused difficulty. Consequently, it was concluded that the significance of the measured values for individual readings was questionable and only the trend of a series of readings could be credited with validity.

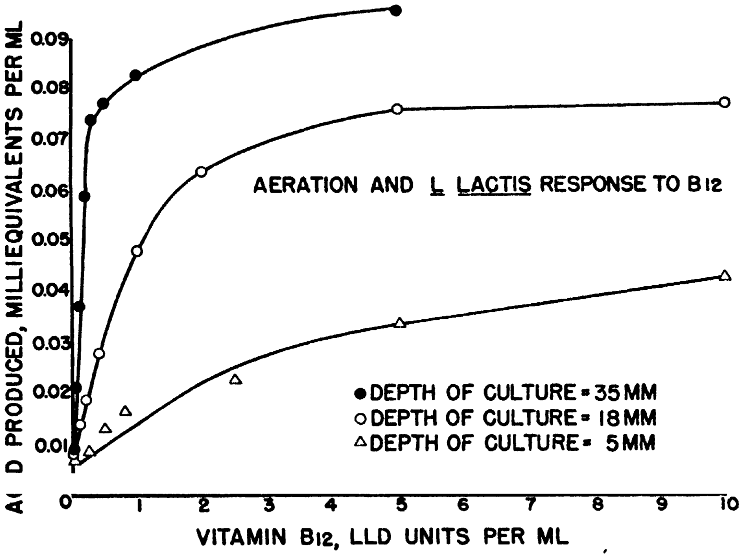

Fig. 1

Measurements on uninoculated medium demonstrated that the oxidation-reduction potential increases with a decrease in pH (16). However, repeated measurements on cultures actively growing under microaerophilic conditions showed a decreasing potential, even though *L. lactis* produces considerable acid during growth. Measurements were taken on uninoculated medium through which oxygen-nitrogen mixtures of varying oxygen concentration were bubbled, and in every series the oxidation-reduction potential of the medium was directly related to the oxygen tension (17). Reducing substances such as ascorbic acid, isoascorbic acid, cysteine, and glutathione markedly lowered the oxidation-reduction potential of the uninoculated medium, whereas oxidizing agents such as hydrogen peroxide, potassium ferricyanide, and potassium permanganate raised the potential.

Of the reducing substances, ascorbic acid and isoascorbic acid were found to be most active in maintaining reduced conditions in the uninoculated medium, and most effective in stimulating growth of *L. lactis* in the absence of the LLD growth factor. Thus, 0.5 mg. per ml. of ascorbic acid[5] lowered the E_h of the uninoculated medium from 0.360 volt to 0.170 volt and always gave maximum growth of *L. lactis* in assay tubes, even when autoclaved with the medium. Smaller concentrations of ascorbic acid failed to produce as low a potential, and growth response in the absence of the LLD factor was erratic. Glutathione was observed to undergo oxidation during measurement and as much as 5.0 mg. per ml. of medium was required to reduce the oxidation-reduction potential of the uninoculated medium to the same extent and allow maximum growth of *L. lactis* in the absence of added LLD growth factor. Lower concentrations of this reducing agent were ineffective in substituting for the growth-promoting properties of liver extract or vitamin B_{12} under the same conditions.

Inhibition of growth was noted when oxidizing agents were added to cultures. Hydrogen peroxide proved the most effective, but its inhibitory effect was overcome by the LLD factor, as will be shown in a subsequent section. A similar relationship was noted with potassium permanganate, but larger quantities of this oxidizing agent were required.

An interesting exception to these phenomena was observed in the effect of potassium ferricyanide. This oxidizing agent failed to inhibit growth and instead had a stimulatory effect in lower concentrations in the absence of liver extract or vitamin B_{12}. Similar stimulation has been noted by Knaysi and Dutky with *Clostridium* (19). They likewise noted the relative ineffectiveness of potassium ferricyanide in maintaining inhibitory oxidation-reduction potential levels.

Hydrogen Peroxide

Since lactobacilli are reported to be catalase-negative (20), the observation that hydrogen peroxide is highly inhibitory to the growth of *L. lactis* was of considerable interest. For these studies, a modification of the potato peroxidase method of Main and Shinn (21) proved to be a simple and quantitative method for the determination of peroxide. Small uniform cylinders of fresh potato were found to be more satisfactory than potato extract. With a small reaction volume (1 ml.) and an accurately controlled reaction time (10 minutes) the blue color which developed in the presence of *o*-tolidine could be measured colorimetrically (Evelyn photoelectric colorimeter fitted with a 660 mμ filter) with a precision among replicates of ±10 per cent, in a range of 10 to 50 parts per million of H_2O_2.

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[5] Shive (18) has reported briefly on ascorbic acid substitution for vitamin B_{12}.

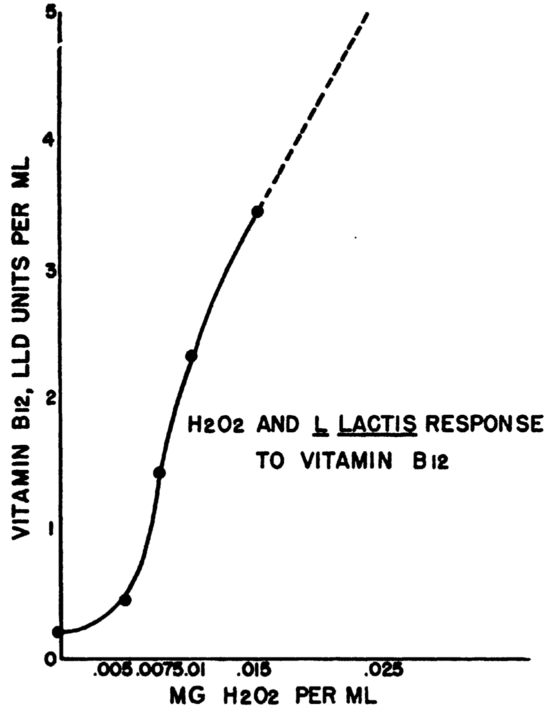

FIG. 2. The points represent vitamin B_{12} required for half maximal growth at each H_2O_2 level.

Standard curves were prepared with H_2O_2 in water solution or in culture medium.

With this method, it was possible to follow the stability of H_2O_2 added to medium and to test cultures for peroxide production. In spite of the rather rapid decomposition of H_2O_2 when added to autoclaved medium, it was found that 10 p.p.m. of added H_2O_2 were definitely inhibitory to the growth of *L. lactis* in assay tubes. The addition of crystalline vitamin B_{12} overcame this inhibition (Fig. 2). The initial lag in the curve relating peroxide addition to vitamin B_{12} requirement is very likely due to destruction of low concentrations of peroxide through interaction with medium constituents.

The activity of vitamin B_{12} in overcoming the H_2O_2 inhibition cannot be attributed to H_2O_2 destruction, because no discernible difference in recovery of added H_2O_2 was found in medium with and without vitamin B_{12}. Furthermore, it is possible to demonstrate appreciable peroxide formation by *L. lactis* in shallow layer cultures containing as much as 0.1 γ of vitamin B_{12} per ml. Sterile medium with or without vitamin B_{12}, under the same conditions, shows no peroxide. Actively growing anaerobic or deep layer cultures, which do not require added LLD factor, likewise do not show any peroxide.

To investigate further the relationship between H_2O_2 and the LLD factor, the effect of the removal of peroxide in shallow layer cultures was determined. This was accomplished by aseptic addition of appropriate quantities of neutralized pyruvic acid, which had been sterilized by filtration through a sintered glass filter. By testing for the peroxide content of such treated cultures throughout incubation, it was established that approximately 6 mg. of pyruvic acid per ml. of culture effectively destroyed all peroxide formed in actively growing shallow layer cultures. Table II demonstrates that such destruction of peroxide results in effective increase in acid production in response to a given concentration of vitamin B_{12}. Turbidity readings, not shown in Table II, show a corresponding increase in growth. Lower concentrations of pyruvic acid failed to show complete destruction of peroxide, and the response of *L. lactis* to the same concentration of vitamin B_{12} in these cultures was correspondingly lower. In shallow layer cultures, pyruvic acid failed to stimulate *L. lactis* in the absence of vitamin B_{12}.

TABLE II

Effect of Pyruvic Acid on Response of L. lactis to LLD Factor in Shallow Layer Culture

Pyruvic acid	Titer* in response to 5 units vitamin B_{12} per ml.	H_2O_2
mg. per ml.		*mg. per ml.*
0	2.9	>0.05
0.13	3.9	0.040
0.64	5.8	0.036
1.3	6.8	0.020
6.4	7.9	0.010
13.0	5.8	<0.010
	In absence of vitamin B_{12}	
0	0.9	<0.010
6.4	1.3	<0.010

* Acid (in 10^{-4} gm. molecule) produced in 10 ml. in 3 days.

As a more specific agent for the destruction of peroxide, catalase was substituted for pyruvic acid. If the catalase is introduced at the beginning of the logarithmic growth phase, even the short period of effective peroxide destruction produced by the catalase (4 to 7 hours) is sufficient to result in a significant increase in response of the organism to a given concentration of LLD factor. Since crystalline catalase was not available, it was not possible to add amounts sufficient to destroy peroxide during the whole incubation period, and the increase in growth and acid production was therefore never as great as that obtained with pyruvic acid. Like pyruvate, in shallow layer cultures the catalase extract failed to produce stimulation in the absence of LLD factor.

Unlike pyruvate, which not only destroys peroxide, but also reduces the oxidation-reduction potential of the medium by other reactions, catalase is only effective in destruction of peroxide. In assay tube controls, where peroxide formation cannot be demonstrated, only pyruvate allows maximum growth of *L. lactis* in the absence of LLD factor. In this respect pyruvate resembles the action of ascorbic acid and glutathione.

These experiments established the similarity between the increased LLD factor requirement of *L. lactis* artificially produced by the addition of H_2O_2 to assay tube cultures and the increased LLD factor requirement observed in aerated cultures where peroxide is produced by the organism. Although the relationship between the LLD factor requirement of the organism and the peroxide concentration of the medium is clear, the nature of the mechanism involved requires further elucidation. It is suggested that the observed phenomena involve two metabolic processes: (1) the production of reducing conditions in the culture medium as a result of rapid growth of *L. lactis*, such growth being stimulated at high oxidation-reduction potentials by vitamin B_{12} or other sources of the LLD factor; (2) the production of H_2O_2 when growth takes place under aerated conditions. This raises the oxidation-reduction potential of the medium and results in cessation of further growth unless more LLD factor is available. The LLD factor fails to overcome peroxide inhibition when the concentration of the latter is too great.

The growth of the organism under given conditions is determined by the relative rates of these processes. The rates can be shifted by the addition of agents which affect the concentration of peroxide, by the addition of oxidizing or reducing agents, by increasing or lowering the oxygen tension, and by other environmental factors which influence the oxidation-reduction potential of the culture.[6]

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[6] Since these data were prepared for publication, similar effects of aeration and reducing substances have been reported (Kocher, V., *Internat. Z. Vitaminforsch.*, **20**, 369 (1949)).

SUMMARY

Data have been presented to show that the requirement of *Lactobacillus lactis* Dorner for the LLD factor is related to environmental factors as follows:

1. *L. lactis* fails to grow in the absence of CO_2 even when the LLD growth factor is present in the medium.
2. In the presence of CO_2, anaerobic conditions produced by the addition of reducing substances, or removal of oxygen from the atmosphere, or other factors which lower the oxidation potential of the medium, eliminate the requirement for the LLD factor.
3. Aeration or oxidizing agents inhibit *L. lactis* growth. This inhibition is overcome by the LLD growth factor.
4. The requirement for LLD under aerobic conditions is related to the peroxide content of cultures.

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A SIMPLE METHOD FOR DETERMINING SERUM COPPER*

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The determination of serum copper is of value in a number of metabolic studies, particularly in the investigation of the anemia of chronic infection. Presented here, together with illustrative data, is a method for determining copper in serum, based on the direct extraction of copper carbamate from serum with isoamyl alcohol. Burch *et al.* in their method for serum iron have demonstrated that such direct extractions with immiscible solvents are satisfactory (1). Simplicity of methodology is the chief advantage of this procedure; steps such as dry ashing, wet oxidation, and multiple extractions with trichloroacetic acid are eliminated.

Sodium diethyldithiocarbamate, the reagent used to react with copper in this procedure, gives colored complexes with a number of heavy metal cations (2). In biological material, however, iron is the chief interfering substance. This interference of iron is satisfactorily eliminated by developing the copper carbamate complex in an ammoniacal solution containing pyrophosphate and having a pH 9 or greater (2–4). The color intensities of other interfering metals such as nickel and cobalt are weight for weight only one-twentieth to one-thirtieth as great as that given by copper (5). Because the concentration of these ions (Ni and Co) in serum is of such a relatively low order, their interference is negligible.

The final color of the extracted copper carbamate is the result of three contributing components: serum copper, copper present as impurity in the reagents, and substances other than copper carbamate which are extracted from serum by isoamyl alcohol.

Copper carbamate is soluble in a number of organic solvents in addition to isoamyl alcohol, namely ethyl ether, amyl alcohol, amyl acetate, bromobenzene, and carbon tetrachloride (2). Of these reagents, only ethyl ether and isoamyl alcohol were tried as extractives. The latter proved to be more satisfactory.

Reagents and Apparatus—

1. Sodium pyrophosphate. Saturate redistilled water with the salt.
2. Ammonium hydroxide, 10 to 12 per cent. Distil 28 per cent reagent

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* The opinions expressed in this paper are those of the author, and do not necessarily represent the official views of any governmental agency.

into redistilled water to saturation. Dilute to the required concentration with redistilled water.

3. Sodium diethyldithiocarbamate, 2 per cent aqueous solution. Remove the copper by shaking with carbon tetrachloride which has been distilled over calcium oxide (6). The solution is stable for some weeks if it is stored in the dark (2).

4. Isoamyl alcohol. Distil from an all-glass apparatus, saturate with water, and store at 4°.

5. Metal-free water. Distilled water, redistilled from a resistant glass apparatus, should be used in the preparation of all reagents and in the final rinsing of the equipment.

6. Standard copper solution. Prepare the stock solution by dissolving 0.3928 gm. of uneffloresced $CuSO_4 \cdot 5H_2O$ in redistilled water and diluting to 1 liter. From this stock solution the working standards may be prepared. It is best to use a solution containing 1 γ per ml. because the final volume of the aqueous phase should be kept at a minimum.

7. Test-tubes. 13 × 100 mm., with ground glass stoppers.

8. Pipettes. Serological, 0.2 ml. graduated to 0.01 ml.

9. Mechanical shaker, Kahn.

10. Spectrophotometer. In this laboratory a Coleman model No. 6, equipped with 12 × 75 mm. cuvettes, was used.

Method

Into each of two glass-stoppered test-tubes deliver exactly 1.0 ml. of serum. Add to each of the tubes 0.2 ml. of saturated solution of sodium pyrophosphate; mix by tapping gently. To each of the tubes add exactly 0.04 ml. of 10 to 12 per cent ammonium hydroxide. Again mix by tapping.

Add 0.2 ml. of sodium diethyldithiocarbamate to one of the tubes; the other is the serum blank. Mix and allow to stand for 1 hour. Deliver exactly 3.0 ml. of isoamyl alcohol into each tube. Place tubes in a Kahn rack, and securely fasten them into a horizontal position on a mechanical shaker. (It is convenient to place a sponge against one end of the shaker and clamp the rack so that the stoppered ends of the tubes are firmly pressed against the sponge.) Shake for 15 minutes.

Place the tubes in centrifuge cups and cool until the water used in balancing the cups begins to form crystals of ice.

Centrifuge at 4000 R.P.M. for 10 minutes. If the minimum required volume (1.5 ml. for a 12 × 75 mm. cuvette) of extract is not obtained, gently shake the precipitate free into the isoamyl alcohol and repeat the centrifugation.

Carefully pipette the isoamyl alcohol fractions into 12 × 75 mm. cuvettes, stopper, and measure optical densities at 440 mμ. (If cloudy extracts are obtained, place the cuvettes for a few seconds in water warmed to 50–60°.

It is necessary to prepare a reagent blank, substituting distilled water for serum, for each series of determinations. Very low readings, 99 to 100 per cent transmission, are obtained.

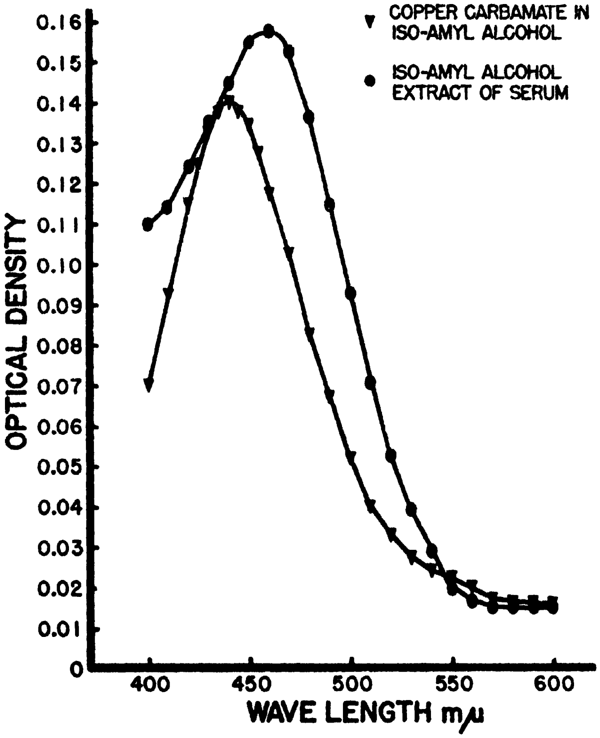

Fig. 1. Absorption curves for copper carbamate and for isoamyl alcohol extract of serum. The copper carbamate curve was obtained by measuring the optical density of 2.5 γ of Cu^{++}, as carbamate, extracted into isoamyl alcohol. 1 ml. of serum was used in the extraction from which the curve was obtained. For both curves the materials were treated as outlined in the procedure recommended in the text.

Calculations—The optical density of the copper carbamate extracted from serum is designated O_{cc}, that for the serum blank O_s, and that for the reagent blank O_r. Serum copper, O_{Cu}, is obtained by calculation:

$$O_{cc} - (O_s + O_r) = O_{Cu}$$
$$O_{Cu} \times K \times 100 = \text{copper, } \gamma \text{ per cent}$$

K is determined from the calibration curve (Fig. 2). In our work K is equal to 17.48.

EXPERIMENTAL

As previously mentioned, the final color is due to three components. A large percentage of the density of the final color is due to materials extracted from the serum other than copper carbamate. This point is demonstrated in Fig. 1, which shows that the maximum light absorption for both an isoamyl alcohol extract of copper carbamate from aqueous solution and an isoamyl alcohol extract of the serum blank occurs in the same general region of the spectrum.

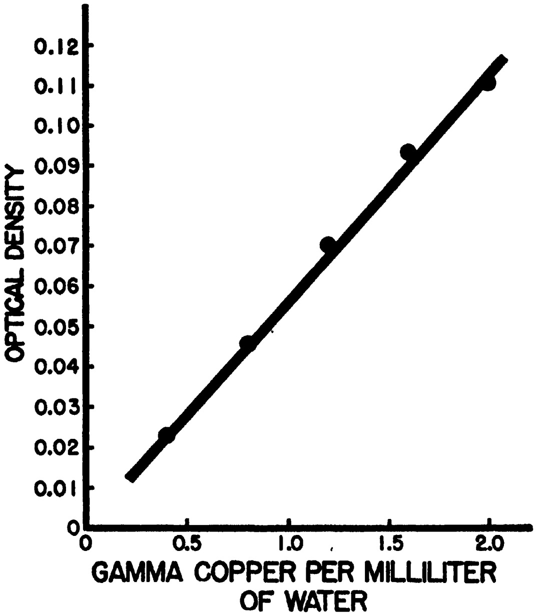

FIG. 2. Calibration curve obtained by extracting copper carbamate from aqueous solution with isoamyl alcohol. The conditions of extraction were the same as those outlined in the text, with measurements at a wave-length of 440 mμ. The direct proportionality is shown between the concentration of copper and the optical density.

The color concentration relationship of copper carbamate in isoamyl alcohol is linear up to a concentration of about 2 γ (Fig. 2). Above this value, however, there is an appreciable deviation from Beer's law. This should be borne in mind, particularly when performing recovery experiments; and in such experiments, concentration figures should be obtained from a calibration curve rather than by use of the calibration constant.

As stated at the outset, it is necessary to have the pH adjusted to 9 or greater, in order to prevent the interference of iron. Six different serums, treated according to the method above, gave pH readings from 9.88 to 10.01.

TABLE I

Recovery of Copper Added to Serum

Experiment No.	Cu in serum	Cu added	Total Cu	Cu found	Recovery
	γ per 100 ml. serum	*γ per 100 ml. serum*	*γ per 100 ml. serum*	*γ per 100 ml. serum*	*per cent*
1	118	50	168	169	102
2	118	50	168	169	102
3	112	50	162	160	96
4	112	50	162	162	100
5	125	50	175	171	92
6	125	50	175	171	92
7	118	100	218	214	96
8	118	100	218	214	96
9	112	100	212	210	98
10	112	100	212	206	94
11	125	100	225	221	96
12	125	100	225	221	96
13	118	150	268	278	106
14	118	150	268	273	103
15	125	150	275	278	102
16	125	150	275	275	100
Average					98

TABLE II

Comparison of Determination of Copper with Present Method and Trichloroacetic Acid Extraction Method

Serum No.	Present method	Method of Cartwright *et al.*	Difference
	γ per 100 ml. serum	*γ per 100 ml. serum*	
1	196	208	−12
2	200	197	+3
3	143	152	−9
4	88	94	−6
5	88	94	−6
6	88	94	−6
7	194	190	+4
8	163	168	−5
9	115	115	0
10	156.	151	+5
11	138	134	+4
Average			−2.5

Experiments show a fairly consistent and quite satisfactory recovery of added copper (Table I). In order to check the values obtained by this method against those of another, the procedure of Cartwright *et al.* (7), which has been in use in this laboratory for the past year, was chosen. The results are presented in Table II. All analyses were performed on serum from subjects who had fasted 12 hours.

Determinations were performed on three groups of subjects: apparently healthy males, apparently healthy females, and male patients with anemia of chronic infection (osteomyelitis). The results are tabulated in Table III. The averages for the control groups are less than those reported by Cartwright *et al.* (7) for their method (males 116 γ per cent, females 131 γ per cent). However, utilizing this same procedure, we obtained an average of 92 γ per cent (the same for the present method) on a group of ten apparently healthy males.

TABLE III

Serum Copper Values in Three Groups of Subjects

Serum specimen No.	"Normal" males	"Normal" females	Males with anemia of chronic infection
	γ per 100 ml. serum	*γ per 100 ml. serum*	*γ per 100 ml. serum*
1	106	79	133
2	73	100	190
3	112	97	168
4	92	97	175
5	64	120	124
6	112	125	208
7	88	94	149
8	110	119	147
9	76		
10	92		
Average.........	92	104	162

In an attempt to determine the individual variation of the serum copper, determinations were made at weekly intervals for a period of 1 month on four apparently healthy individuals (two males and two females). In the males, the differences between the lowest and highest values obtained were 8 and 19 γ per cent; for the females, 22 and 24 γ per cent.

SUMMARY

A simple method for determining serum copper, based on the extraction of copper carbamate directly from serum with isoamyl alcohol, is presented. Recovery experiments compare favorably with more complex procedures.

The average value for males is 92 γ per cent; for females the average is

104 γ per cent. The largest individual variation in serial determinations was 24 γ per cent in a female subject.

I wish to express my appreciation to Dr. R. E. Johnson and Dr. F. Sargent for assistance in preparing this report.

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RELATIVE VITAMIN E POTENCY OF NATURAL AND OF SYNTHETIC α-TOCOPHEROL*

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A reasonably accurate estimate of the relative physiological value of natural and of synthetic α-tocopherols is needed by the clinician, the pharmaceutical industry, and the experimental nutritionist to facilitate their use of vitamin E in a more quantitative manner. At present there is preliminary information available for two species of animals. It was tentatively established (1) in 1944 that for rats natural *d*,α-tocopherol possessed 50 per cent more potency than synthetic *dl*,α-tocopherol, with resorption during gestation as a criterion. In 1947 Hove and Harris reported (2) that for rabbits natural α-tocopherol was 22 per cent more active than the synthetic racemic form, based on effectiveness in curing muscle dystrophy.

It is now possible to reevaluate the relative potency of *d*- and of *dl*,α-tocopherol in the rat, based on the results of numerous bioassays in this laboratory since 1944, by means of an improved assay procedure (3). From these data a new value of 1.36, for the ratio of the potency of natural tocopherol to the potency of synthetic tocopherol, has been obtained.

EXPERIMENTAL

The bioassay procedure of Mason and Harris (3) was used, except that variations in diet were made as shown in Table I. Statistical treatment of the results was based on procedures outlined by Miller *et al.* (4).

The natural tocopherols used were all pure compounds, crystals in the case of the esters, and were kindly supplied to us by the Chemical Plant and the Organic Chemistry Department of Distillation Products, Inc. The synthetic tocopherol in these experiments was the international standard, *dl*,α-tocopheryl acetate.

Results

Table II shows the relationships obtained during a 3-year period whenever synthetic and natural tocopherol were included in the same bioassay. When esters were used, it was found easier to handle the results by recording the doses as the weight of the tocopherol, calculated stoichiometrically from the weight of the ester. For example, a dose of 1.099 mg. of α-tocopheryl acetate is tabulated as 1.000 mg. of α-tocopherol, as ester.

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* Communication No. 146.

The individual values for the ratio of the relative potency of natural *versus* synthetic tocopherol vary quite widely, from 1.03 to 1.81. However, these values are all homogeneous within the limits indicated by a χ^2 test. The limits of uncertainty for the individual comparisons also vary, as shown by the standard errors of the ratios, which range from 0.22 to 0.44.

TABLE I

Composition of Diets Used in Bioassays

Ingredients	Diet 30	Diet 39	Diet 301
	per cent	*per cent*	*per cent*
Casein, crude	20	20	22
Vitaminized casein*			2
Corn-starch	54	61	
Cerelose			60
Yeast, dried brewers'	10	10	
Salt Mixture 2, U. S. P.	4	4	4
Lard	12		12
Stripped corn oil†		5	
Vitamins A and D‡			

* Crude casein with the following crystalline vitamins added in amounts such that the final ration contained thiamine hydrochloride 10 γ per gm., riboflavin 10 γ per gm., pyridoxine 10 γ per gm., niacin 20 γ per gm., Ca pantothenate 25 γ per gm., 2-methyl-1,4-naphthoquinone 50 γ per gm., inositol 500 γ per gm., and choline chloride 1 mg. per gm.

† Distilled on a molecular still to remove vitamin E.

‡ Added to the fat component of the diet as natural vitamin A ester concentrate and vitamin D_2 concentrate to supply 40 units of vitamin A and 4 units of vitamin D per gm. of final ration.

These variations were taken into consideration in determining a weighted mean for the ratios and also a standard error of the weighted mean. The value of 1.36 ± 0.07 represents the best estimate from these data of the relationship between the potency of natural α-tocopherol *versus* synthetic α-tocopherol. The standard error of the weighted mean, ±0.07, indicates that in another similar series of comparisons between the two types of α-tocopherol the weighted mean would fall within 1.29 and 1.43, with a probability of 2 out of 3.

It should be noted that this relationship in potency, 1.36, applied both to the comparison between free *d*,α-tocopherol and free *dl*,α-tocopherol and to that between esterified *d*,α-tocopherol and esterified *dl*,α-tocopherol.

TABLE II

Relative Vitamin E Potency of Natural and Synthetic α-Tocopherols

Assay date	Diet No.	No. of animals	Median fertility dose*		Ratio M. F. D. *dl*,α-tocopherol / M. F. D. *d*,α-tocopherol	±s.e. of ratio‡
			Natural tocopherol†	Synthetic tocopherol		
***d*,α-Tocopheryl esters *vs.* *dl*,α-tocopheryl acetate (international standard)**						
			mg.	*mg.*		
Jan., 1945	30	30	0.54	0.84	1.56	0.36
Apr., 1945	39	36	0.67	1.05	1.57	0.36
Sept., 1945	39	38	0.47	0.65	1.38	0.27
Nov., 1945	30	36	0.42	0.76	1.81	0.43
May, 1946	301	48	0.61	0.75	1.23	0.26
July, 1946	301	36	0.50	0.57	1.14	0.26
Sept., 1946	301	62	0.44	0.72	1.64	0.42
Nov., 1946	301	55	0.38	0.53	1.39	0.37
Feb., 1947	30	38	0.40	0.41	1.03	0.39
" 1947	301	25	0.43	0.52	1.21	0.25
Apr., 1947	301	49	0.37	0.42	1.14	0.44
June, 1947	301	51	0.40	0.61	1.53	0.22
July, 1947	301	35	0.43	0.46	1.07	0.25
Sept., 1947	301	45	0.45	0.53	1.18	0.31
Oct., 1947	301	45	0.34	0.54	1.59	0.28
***d*,α-Tocopherol *vs.* *dl*,α-tocopherol (Merck)**						
Feb., 1946	301	49	0.60	0.81	1.35	0.26
" 1947	30	36	0.44	0.60	1.36	0.40
Weighted mean ± s.e.§					1.36 ± 0.07	

* The weights of tocopherol were calculated stoichiometrically from the weights of the esters.

† The first sample was pure *d*,α-tocopheryl acetate. All others were pure *d*,α-tocopheryl succinate.

‡ Standard error of the ratio, or of the comparison, between M. F. D. values =

$$\pm\sqrt{\left(\frac{\text{S.E.}_{d}}{\text{M. F. D.}_{d}}\right)^2+\left(\frac{\text{S.E.}_{dl}}{\text{M. F. D.}_{dl}}\right)^2}$$

(See Arkin and Colton (6).) S.E._{d} and S.E._{dl} are standard errors of the M. F. D. values of *d*,α-tocopherol and *dl*,α-tocopherol respectively. They are calculated from the dose-response curve, as illustrated by Miller and Tainter (7). The standard error of an M. F. D. represents the range within which the M. F. D. value will fall in 2 out of 3 subsequent bioassays.

§ Weighted mean = $\Sigma(W_R \times \text{ratio})/\Sigma(W_R)$, where $W_R = 1/(\text{S.E. of ratio})^2$ (Mason and Harris (3)). The standard error of the weighted mean = $\pm\sqrt{1/\Sigma(W_R)}$ (Mason and Harris (3)).

Since *dl*,α-tocopheryl acetate has a potency of 1.0 international unit per mg. by definition (5), it is now possible to express an international unit equivalency for *d*,α-tocopheryl esters. 1 mg. of *dl*,α-tocopheryl acetate = 1.00 I.U., or 1 mg. of *dl*,α-tocopherol, as an ester, = 1.10 I.U. Since the *d* isomer is 1.36 times as active as the *dl* form, 1 mg. of *d*,α-tocopherol, as ester, = 1.10 × 1.36, or 1.50 I.U.

The relatively great variation in median fertility dose from one bioassay to another, while having no bearing on potency ratios, is a bothersome factor in the bioassay procedure and deserves some discussion here. It is evident that in a method which requires 0.34 mg. of pure vitamin E one time (assay of October, 1947) and 0.67 mg. of the same material another time (assay of April, 1945) to induce the same physiological response there are factors of diet, of technique, or of animals which are not being maintained constant from one bioassay to another. We have tried to eliminate this variation in values of median fertility dose between assays without success so far. Diet ingredients have been purchased in large quantities and so the same ingredients were used in several successive assays. Diets were compounded carefully by weight and frequently (every 4 days) to prevent changes due to long or variable storage. The same individual performed all of the animal manipulations in successive assays and tried to maintain constant each step in the procedure. The rats were produced in our own breeding colony under as uniform conditions as possible. Variations in median fertility dose between assays still occurred and we are continuing the search for the variable factor involved. Table I shows some of the variations in diet which were made during the course of our experiments. The results obtained with each of these diets are not detailed, since they showed that values of the median fertility dose were not influenced by these changes in diet.

SUMMARY

Vitamin E bioassays were carried out in which the potency of natural *d*,α-tocopherol, free and esterified, was compared with comparable forms of synthetic *dl*,α-tocopherol. Prevention of fetal resorption during gestation in rats was used as the criterion of physiological response.

Natural *d*,α-tocopherol was found to be 1.36 (±0.07 standard error) the potency of synthetic *dl*,α-tocopherol. This relationship applied both to the comparison between free *d*,α- and free *dl*,α-tocopherol, and to that between esterified *d*,α- and esterified *dl*,α-tocopherol.

α-Tocopherol, in natural *d*,α-tocopherol esters, can be expressed in terms of international units; *e.g.*, *d*,α-tocopherol, in ester form, is equivalent to 1.5 I.U. of vitamin E per mg.

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LIPOGENESIS FROM GLUCOSE IN THE NORMAL AND LIVERLESS ANIMAL AS STUDIED WITH C^{14}-LABELED GLUCOSE*

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(Received for publication, January 22, 1949)

Since the experimental demonstration that fatty acids can be synthesized from carbohydrate in the animal body, there has been much speculation on the site or sites where this biochemical process occurs (1–7). It is generally accepted that the liver is a site for conversion, but conclusive proof of its occurrence in extrahepatic tissues has yet to be presented. The availability of C^{14}-labeled glucose has made possible a direct study of the incorporation of glucose-C^{14} into the carbon chain of fatty acid molecules. We wish to report here results obtained with both normal and eviscerated animals.

EXPERIMENTAL

Treatment of Mice and Rats before Experimental Run—For 2 or more weeks before the experimental runs, the animals were fed *ad libitum* a high carbohydrate diet composed of 60 per cent glucose monohydrate, 22 per cent casein (Labco, vitamin-free), 6 per cent brewers' yeast, 6 per cent Hawk-Oser salt mixture (8), and 6 per cent Cellu flour. The yeast furnished the only source of fat in this diet. Mice treated as described above were weighed daily, and only those whose weights remained constant throughout a 2 week period were selected for study.

Collection of Expired CO_2—Each mouse was placed in a glass metabolism cage which was ventilated continuously with CO_2-free air at 27–28°. The air collected from the cage was passed through a column of carbonate-free NaOH (17 m.eq. per mouse were used for each hour of CO_2 collection). A porous glass disk at the bottom of the column served to break the stream of air into fine bubbles. The NaOH-Na_2CO_3 mixture obtained was made to volume with CO_2-free distilled water, and two aliquots were used for the determination of its CO_2 content. One was titrated with 0.1 N HCl to the brom cresol green end-point; this provided a measure of the total amounts of Na_2CO_3 and NaOH present. An excess of $BaCl_2$ was added

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* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the Corn Industries Research Foundation.

to the other mixture which was titrated with 0.1 N HCl to the phenolphthalein end-point; this titration represented the unused NaOH. The $BaCO_3$ mixture was centrifuged and the precipitate washed with distilled water. The radioactivity of the $BaCO_3$ was determined as described below.

Evisceration of Rats—The superior mesenteric artery, the celiac axis, and the portal vein were ligated in the order listed. The celiac axis was tied above the origin of the hepatic artery. Ligatures were then placed at the lower ends of the esophagus and rectum, and the entire gastrointestinal tract, spleen, and pancreas were excised. The ligature applied to the rectum was arranged so as to occlude the inferior mesenteric artery. This operation results, essentially, in a functionally liverless rat, for, although the liver remains *in situ*, it is deprived of its portal and hepatic blood supplies.

Extraction of Tissue Fatty Acids—Immediately after their removal from the animal, the tissues were placed in hot 30 per cent KOH solution (2 cc. per gm.) and heated for 18 hours on a steam bath. The mixture was allowed to cool, and extracted three times with a huge excess of petroleum ether. The three petroleum ether extracts were combined and evaporated to a small volume, and the concentrated petroleum ether was washed with distilled water to remove soaps that might have entered it. The water extract was added to the aqueous residue.

The aqueous residue was acidified (pH 3 or lower, as judged by brom cresol green) and its fatty acids extracted thoroughly with petroleum ether. The petroleum ether extracts were combined and made to volume. An aliquot of this solution was transferred to a tared flask and its fatty acid content determined by weight. The fatty acids of another aliquot were oxidized by means of the Van Slyke wet combustion solution (9) and the liberated CO_2 collected as $BaCO_3$. The $BaCO_3$ was mounted on an aluminum plate and its radioactivity measured as described below.

Isolation and Identification of Palmitic Acid—A petroleum ether solution of fatty acids was evaporated and the residue dissolved in methanol. The methanol solution of fatty acids was hydrogenated over platinum oxide at atmospheric pressure. After the catalyst had been removed by filtration, sulfuric acid was added to the methanol solution and the mixture heated under a reflux for 1 hour. The crude methyl esters were isolated in the usual manner and distilled according to the amplified distillation procedure of Weitkamp (10), with "Eureka white oil." A column with packing, as described by Mitchell and O'Gorman (11), was employed for the distillation. The oil-diluted fraction containing methyl palmitate was hydrolyzed by heating with a solution of potassium hydroxide in isobutyl alcohol. The hydrolysate was then acidified and extracted with hexane. The hexane extract was washed with water and then shaken with an excess of aqueous potassium hydroxide. The two layers were not separated, but in order to insure complete conversion of palmitic acid to its potassium salt, the hexane was boiled off. The resulting aqueous solution of potassium palmitate was first washed with hexane to remove the carrier oil and then acidified. It was also warmed to insure complete reaction. The cooled mixture was filtered and recrystallized, once from 50 per cent aqueous acetone and once from hexane.

Determination of C^{14} Content of $BaCO_3$—The $BaCO_3$ was suspended in 95 per cent ethanol and the mixture finely ground. It was then transferred to an aluminum plate where the ethanol was evaporated with the aid of heat from an infra-red lamp. The details of this procedure, as well as the method for the determination of the radioactivity of the mount, have been described by Dauben *et al.* (12).

Incorporation of Glucose-C^{14} into Fatty Acids by Normal Mouse

The experimental runs lasted for 24 or 48 hours during which the mice had access to water and to the high carbohydrate diet described above which had been thoroughly mixed with a sample of C^{14}-labeled glucose.[1] The glucose and C^{14} ingested by each mouse were determined by an analysis of the uneaten portion of the diet.

At the end of the experimental run, the mice were killed with an intraperitoneal injection of nembutal. In Mice 1, 2, 3, 6, and 7, the livers and the carcasses were analyzed separately; in these animals the term carcass, therefore, refers to all tissues with the exception of the liver.

In the case of Mice 4 and 5, separate analyses were made for the liver and small intestine. Thus the term carcass here refers to the whole mouse less these two organs.

The results obtained in seven mice, five of which were sacrificed 24 hours, and two, 48 hours, *after the feeding of the radioglucose was begun*, are recorded in Table I. At the 24 hour interval, 10 to 15 per cent of the glucose-C^{14} was recovered in the fatty acid fraction isolated from the whole animal. The actual amounts of the *ingested*[2] glucose that had been converted to fatty acids were obtained by multiplying the numerical proportions of the C^{14} recovered as fatty acids by the total mg. of glucose ingested. The values are recorded in Table I. Thus in 24 hours, from 175 to 265 mg. of ingested glucose were converted to fatty acids and, in 48 hours, 459 to 680 mg.

The C^{14} incorporated into liver fatty acids accounted for less than 2 per cent of the administered C^{14}. This was true for both intervals studied. The isotope concentrations of the fatty acids of this tissue are of considerable interest and the values (specific activities) are recorded in Table II. The fact that the specific activities of the liver fatty acids so exceed those of the carcass (body minus liver) makes it appear unlikely that the fatty acids of the liver originate in extrahepatic tissues, but, since the specific activities of each of the extrahepatic tissues were not measured separately, this interpretation may be open to question.

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[1] We are indebted to Dr. W. Z. Hassid for the radioactive glucose used in this study.

[2] It should be noted that the mice had access to the diet containing the radioactive glucose throughout the *entire* period of observation, *i.e.*, up to the time they were sacrificed. The unabsorbed glucose was not measured.

TABLE I

Fate of Glucose in Mouse

Mouse No.*	Weight	Fatty acids isolated from mouse	Duration of experiment	Glucose-C^{14} ingested	Per cent of ingested C^{14} recovered as				Glucose ingested†	Ingested glucose converted to fatty acids	Ingested glucose converted to CO_2	Per cent ingested glucose accounted for in CO_2 and fatty acids
					Exhaled CO_2	Fatty acids						
						Liver	Carcass	Total				
	gm.	*mg.*	*hrs.*	*counts per min.* $\times 10^6$					*mg.*	*mg.*	*mg.*	
1 ♂	20	2640	24	0.853	70.5	1.5	8.3‡	9.8	1790	175	1260	80
2 ♂	18	1770	24	1.250	61.5	1.7	8.4‡	10.1	1810	182	1110	72
3 ♂	19	1950	24	1.163	61.8	1.8	10.0‡	11.8	2210	265	1360	74
4 ♀	20	3096	24	0.455	67.6	1.9	12.9§	15.4				82
5 ♀	25	4117	24	0.650	61.5	1.9	12.3§	14.5				75
6 ♂	20	2110	48	1.534	75.8	0.2	12.6‡	12.8	3480	446	2640	89
7 ♂	23	2860	48	1.520	84.5	1.4	14.8‡	16.2	4210	680	3560	101

* Mice 4 and 5 were of the C57 black strain; the rest were of the Lister ABC strain.

† The mice were sacrificed at 6.00 p.m.

‡ All tissues except liver.

§ All tissues except liver and small intestine.

TABLE II

Specific Activity of Fatty Acids in Various Mouse Tissues*

Specific activity† of	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7
Liver fatty acids	185	400	363	112	114	154	353
Intestine fatty acids				44	48		
Carcass fatty acids	27‡	61‡	61‡	20§	20§	92‡	81‡

* Mouse species same as for Table I.

† Specific activity = C^{14} counts per minute per mg. of fatty acids.

‡ All tissues except liver.

§ All tissues except liver and intestine.

The specific activities of small intestine fatty acids were determined in two mice (Nos. 4 and 5) and the results are recorded in Table II. The fact that the values were more than twice those of the carcass implies, as already pointed out by earlier investigators (13), that this tissue is a site for lipogenesis. But the higher specific activities found for liver fatty acids do not exclude the possibility of the liver as a source of intestinal fatty acids.

The mouse fed a high carbohydrate diet and maintained in the steady state oxidized more than 60 per cent of the ingested glucose in 24 hours and in 48 hours (Table I). The percentages oxidized, in addition to those converted to fatty acids, accounted for most of the carbohydrate ingested in 24 and 48 hours.

Conversion of Radioglucose to Fatty Acids by Normal and Eviscerated Rats

Immediately following evisceration and while still under anesthesia, the rats were shaved on an area between the shoulder blades. This area was used for the subcutaneous injection of the C^{14}-containing glucose. Each rat received four injections of the C^{14}-glucose, the first as soon as it had recovered from the anesthetic, the other three at successive intervals of 2 hours. At each interval, the rat was also injected subcutaneously with unlabeled glucose (2 gm. per kilo) in a different region; this administration of carbohydrate served to prevent hypoglycemia. Each rat thus received, at 0, 2, 4, and 6 hours after the functional hepatectomy, two injections of glucose, (1) the C^{14}-labeled glucose, and (2) unlabeled glucose. The rats were sacrificed 2 hours after the last injection.

Two normal rats were used as controls. One of these (Rat T1) was subjected to a sham operation in which the abdominal cavity was opened and the viscera manipulated before the rat was injected with glucose as described above for the eviscerated rat. The other normal rat (No. N1) was not operated on and was injected only with the C^{14}-glucose.

A measure of the unabsorbed C^{14}-glucose was obtained as follows: At the end of the period of observation (*i.e.*, 8 hours after the first injection of the C^{14}-labeled glucose), the rats were anesthetized with ether. Approximately 10 sq. cm. of the skin in the region of the C^{14} injection, and an equal area of the subcutaneous tissues, were excised and homogenized with 10 per cent trichloroacetic acid. The area over the denuded muscle was wiped with cotton and the cotton transferred to the homogenizer. The homogenate was then centrifuged and the precipitate washed. The combined supernatant and washings were analyzed for radioactivity. The amounts of C^{14}-glucose absorbed are recorded in Table III.

8 hours after the first injection, 2 and 3 per cent of the administered glucose-C^{14} were incorporated into fatty acid molecules by the normal rat (Table III). It is clear from Table III that this conversion also takes place in the rat deprived of functioning hepatic tissue. That it proceeds

at an appreciable rate in the functionally hepatectomized rat is the surprising result brought out here.

Proof that the livers of Rats H1 and H2, which remained *in situ*, could not have contributed to the incorporation of the glucose-C^{14} into the fatty acid molecules to any appreciable extent is provided in Table III. Thus only 0.001 per cent of the administered C^{14} was recovered in the fatty acids of the livers of Rats H1 and H2, a value approximately 1 per cent of that found in the liver of the normal rats (Nos. N1 and N2).

Palmitic acid was isolated from Rats H1 and T1 by the method described above. White plates of palmitic acid were obtained whose melting point, 61–62°, is in good agreement with reported values.

TABLE III

Fatty Acid Formation in Normal and Hepatectomized Rats

All observations made 8 hours after first subcutaneous injection of C^{14}-glucose.

Rat No.	Condition	Weight	Fatty acid content of rat	C^{14}-Glucose injected	Per cent of injected C^{14} recovered as fatty acids		Specific activities*	
					Liver	Body	Body fatty acids	Body palmitic acid
		gm.	*gm.*	*counts per min.* $\times 10^6$				
N1	Normal	200	13.5	18.7	0.09	2.0	21	
T1	Operated, control	125†	7.4	18.7	0.10	3.0	79	81
H1	Eviscerated	122†	10.1‡	19.9	0.001	4.2	84	104
H2	"	122†	9.2‡	14.0	0.001	1.5	23	

* Specific activity = C^{14} counts per minute per mg. of fatty acids.

† Preoperative weights.

‡ Fatty acids determined after evisceration.

The pure palmitic acid was oxidized and its specific activity determined. The results are recorded in Table III. In each experiment, the specific activity of the isolated palmitic acid agreed closely with the specific activity of the petroleum ether extract of fatty acids.

DISCUSSION

The experiments that favor the liver as a probable site for the synthesis of fatty acids from carbohydrate have been assembled by Longenecker (2) and by McHenry and Cornett (5). Among these, the experiment carried out by Barrett, Best, and Rideout (1) is significant. They fed rats deuterium-containing fats for 14 days and thereby labeled the body fatty acids. The finding that the subsequent feeding of a deuterium-free, high carbohydrate diet for 7 days resulted in a pronounced dilution of liver fatty acids and little dilution in the body fatty acids led these workers to conclude that the conversion of carbohydrate to fat may have taken place entirely in the liver.

Waelsch, Sperry, and Stoyanoff (14) compared the deuterium concentration of fatty acids in liver, intestine, brain, and carcass in rats whose body fluids had been enriched with deuterium for 4 to 7 days while being fed a diet high in carbohydrate and almost devoid of fat. In the adult rat, the highest atom per cent deuterium was found in the liver; the values for intestine, although lower than those of the liver, were nevertheless much higher than those for brain and carcass. Such observations have also been interpreted to implicate the liver as a site of conversion of carbohydrate to fatty acids.

These same investigators (15) observed that, in the 19 day-old rat which had received heavy water from the 15th to the 19th day of life (*i.e.*, the period of most active myelination), the deuterium concentration of brain fatty acids was 0.24 atom per cent as compared with 0.32 and 0.34 for liver and intestine fatty acids, respectively. The fact that the fatty acid content of the brain doubles in rats between the ages of 15 and 19 days, coupled with the assumption that the breakdown of fatty acids in the young rat brain proceeds at the same slow rate as that in the adult brain, led these investigators to conclude that the deuterium concentration of the brain fatty acids was approximately twice as great as that measured and considerably higher than that found for any of the other tissues of the mothers or of the young rats. They therefore argued that the deposited fatty acids could have originated only in syntheses in the brain itself.

The evidence for the conversion of carbohydrate to fatty acids in the tissues, other than liver, of the *adult* animal is of an indirect nature and hence not conclusive. Tepperman, Brobeck, and Long (4) clearly established that the R.Q. of eviscerated rats, previously trained to consume their daily ration in 3 hours, rises above unity. These rats received both glucose and insulin immediately following evisceration. The Yale investigators suggested that the conversion of carbohydrate to fatty acids was chiefly responsible for these high R.Q. values. It has been recognized for some time, however, that R.Q. values observed after evisceration must be viewed with caution as indicators of the nature of the metabolic activity of this preparation (16), a fact fully recognized and evaluated by Tepperman *et al.* in their studies. The R.Q. data obtained for surviving slices of adipose tissue have similarly been interpreted to indicate an extrahepatic conversion of carbohydrate to fatty acids (7). Suggestive evidence that fatty acid synthesis can occur in the brain of the 40 day-old rat, but at a slow rate, has been presented by Waelsch *et al.* (17), and recently a claim for such a synthesis in adipose tissue has been made by Shapiro and Wertheimer (18).

In the adult rat which had received a high carbohydrate diet and heavy water, Bernhard and Bullet (13) found an occasional higher value for the deuterium concentration of intestinal fatty acids than for any of the other tissues examined. They therefore stated that the intestine is a site of fatty acid synthesis. But their observations have not been confirmed here nor by Waelsch *et al.* (14).

As judged by the results presented here, the conversion of glucose to fatty acids is not limited to a single tissue in the adult rat. Its occurrence in the liver is implied by the higher specific activities of fatty acids in the liver than in the carcass. The isolation of C^{14}-containing palmitic acid from the eviscerated rat that had been injected with C^{14}-glucose can leave no further doubt that the conversion of carbohydrate to fatty acids proceeds in one or more of the extrahepatic tissues. But the results presented here should not be interpreted as minimizing the importance of the liver in this conversion.

The large amounts of glucose-C^{14} incorporated into fatty acid molecules show that conversion to fatty acids is a significant metabolic pathway for ingested glucose. This has already been pointed out by Pauls and Drury (19) and by Stetten and Boxer (20). The values reported in our investigation, namely 10 to 15 per cent in 24 hours and 12 and 16 in 48 hours, for the conversion of the *ingested*[2] glucose-C^{14} to fatty acids are minimum values, since they do not take into account the amount of C^{14}-labeled fatty acids that were degraded during these periods.

SUMMARY

The conversion of carbohydrate to fatty acids was studied in the mouse and rat with C^{14}-labeled glucose.

1. In the mouse maintained on a high carbohydrate, fat-free diet, 10 to 15 per cent of the ingested glucose-C^{14} was incorporated into fatty acids in 24 hours, and 12 and 16 per cent in 48 hours.

2. In this mouse, more than 60 per cent of the ingested glucose-C^{14} was eliminated as CO_2 in 24 and 48 hours. Thus in the fed mouse, nearly all of the carbon of the ingested labeled glucose was accounted for by fatty acids and CO_2.

3. The specific activities of the fatty acids of liver and small intestine were much higher than that of the rest of the mouse. The finding of the highest values in the liver (3 times those of the small intestine and about 6 times those of the carcass) is interpreted to favor the liver as a *primary* site of synthesis of fatty acids from glucose.

4. In 8 hours, about 2 per cent of subcutaneously injected glucose-C^{14}

was converted to fatty acids by the normal rat. C^{14} was also recovered in fatty acids of the rat that had been deprived of both liver and gastrointestinal tract.

5. C^{14}-containing palmitic acid was isolated from eviscerated as well as normal rats that had received C^{14}-labeled glucose. The finding of an appreciable amount of C^{14} in the palmitic acid isolated from the eviscerated rat demonstrates that the conversion of carbohydrate to fatty acids proceeds significantly in tissues other than the liver and intestine.

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THE DETERMINATION OF FUMARIC ACID IN ANIMAL TISSUES BY PARTITION CHROMATOGRAPHY

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The present investigation arose from a need for a method of analysis that would permit the estimation of the small quantities of fumaric acid alleged to be present in animal tissue. Annau *et al.* (1) suggested the possible metabolic significance of fumaric acid, and the "citric acid" cycle of Krebs (4), which followed promptly the demonstration by Orten and Smith (9) of citric acid precursors in the dog, attracted attention to the biological importance of certain other related organic acids. Although schemes of analysis for fumaric acid exist, the early methods either lacked sensitivity or specificity or required equipment which prevented their wide application. The methods of Annau *et al.* (1) and of Massart and van Grembergen (8) employed an extraction of the acid and final volumetric measurement with standard potassium permanganate solution, whereas Krebs and coworkers (5) measured the fumaric acid after reduction to succinic acid. Szegedy (11) used addition reactions with bromine, while Stotz (10) measured the mercury held in mercurous fumarate precipitates. Polarographic methods were developed by Giovanni and Rao (2) and by Warshowsky and coworkers (12). The method of Isherwood (3) for the determination of organic acids in fruits suggested the possibility of adapting the chromatographic technique to this problem. The theory of chromatography developed by Martin and Synge (7) provided direction for the investigation from which evolved the method herein described. This procedure, in our hands, also appears suitable for succinic, malic, and citric acids as well. The present report presents the detailed procedure for the measurement of fumaric acid which has been employed in our laboratory (Marshall, Orten, and Smith (6)).

The principle of the method involves the transfer of the organic acids of the sample to a non-aqueous phase, an amyl alcohol-chloroform mixture, which is then passed through a column of specially treated silica gel; a physicochemical separation of the organic acids is thus accomplished. As will be described in detail later, further separation of fumaric acid from other organic acids, which are released from the column at nearly the same time, can be accomplished on rechromatographing the effluent and using a lower proportion of amyl alcohol to chloroform. The final measurement involves titration with standard alkali of fractions of the effluent and the values obtained describe a curve which approximates the normal curve of error. The reproducible location of the mode of this curve at the same position on the abscissa under comparable experimental conditions demonstrates the specificity of the method. A colorimetric method (Marshall, Orten, and Smith (6)) shown to be specific for fumaric acid, though less sensitive, was employed as a further procedure for verification.

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* The data in this paper have been taken from the dissertation submitted by Lawrence M. Marshall for the degree of Doctor of Philosophy, Wayne University, 1949. Junior Fellow, National Institute of Health, 1946–48. Present address, Howard University School of Medicine, Washington.

Reagents—Acid acetone. 6 ml. of 10 N sulfuric acid diluted to 1 liter with acetone.

Amyl alcohol, tertiary. Eastman; sp. gr., 20/20, 0.815.

Amyl alcohol-chloroform mixtures. The calculated volume of amyl alcohol diluted to 1 liter with chloroform.

Butanol (normal). Baker's Analyzed; sp. gr., 25°, 0.8080 (butyl alcohol, normal).

Butyl alcohol-chloroform mixtures. The calculated volume of butyl alcohol was made up to 1 liter with chloroform.

Chloroform. Baker and Adamson; sp. gr., 20/20, 1.490.

Fumaric acid. M.p. 286°; neutralization equivalent 58; sublimes at 200°. Chas. Pfizer and Company, Brooklyn, New York.

Silica gel. Commercial sodium silicate was allowed to stand for 10 to 24 hours under 4 liters of water. The solution was filtered and an approximate value for total solids was obtained; this guided the dilution so that the sodium silicate solution had a final concentration of 20 ± 2 per cent (weight per solution volume). Several drops of methyl orange were added and 10 N hydrochloric acid was added in a fine stream until the indicator became pink. 150 to 200 ml. were then added in excess and the mixture was allowed to stand for 3 hours, after which it was filtered on a Büchner funnel. The gel was suspended in 3 liters of 10 N hydrochloric acid overnight, filtered by suction, and washed with 5 liters of 5 N hydrochloric acid, 10 liters of distilled water, 10 liters of absolute alcohol, and finally 5 liters of dry ether. The powder was dried in a warm current of air and, after standing 2 weeks, was suspended in 4 liters of 10 N hydrochloric acid and allowed to stand overnight. The material was filtered by suction, washed with 5 liters of 5 N hydrochloric acid, 50 liters of water, and 10 liters of absolute alcohol containing 1 per cent (by weight) of 10 N sulfuric acid. Washing with ether completed the process. The gel was air-dried and before using was dried for a minimum of 24 hours over phosphorus pentoxide. That silica gel which passed, with gentle brushing, through a No. 90 mesh sieve was used.

Thymol blue indicator. 40 mg. of thymol blue were triturated in a mortar with 4 ml. of 0.1 N sodium hydroxide. The solution was washed into a 200 ml. volumetric flask, made to volume with water, and filtered.

Procedure

Calibration—3 ml. of a solution of 0.5 N sulfuric acid are added to 3 gm. of dried silica gel. To this mixture are added 35 ml. of chloroform and the suspension is introduced into an 8 mm. glass tube, 1 meter in length, and closed at the bottom with a cotton plug fitted into a slightly constricted end. Chloroform is allowed to pass through the tube until the column settles as a contiguous mass. The surface of the column is kept moist with 1 ml. additions of chloroform. When the column settles as a single mass, the last portion of chloroform on the surface of the column is allowed to disappear and immediately 1 ml. of a 1 mg. per cent solution of pure fumaric acid in a 10 per cent amyl alcohol-chloroform mixture is added. Immediately after the pure fumaric acid solution has drained into the column, 1 ml. of 5 per cent amyl alcohol-chloroform is introduced; two additional separate portions are added, each being allowed to drain into the column. When the last added portion disappears, the receiver is changed to a 25 ml. test-tube in a rack which contains thirteen (or more) similar test-tubes, the tubes being identified by number, *i.e.* Tube 1, 2, 3, etc. The column is quickly filled with 5 per cent amyl alcohol-chloroform, the meniscus of which becomes the zero on a movable "volume flow" scale placed against the column. The collecting tubes are changed so that the numbers on the receivers correspond to the interval opposite the falling meniscus, *i.e.* Tube 2 at scale interval 2, Tube 3 at scale interval 3, etc. The scale is prepared from wood strips having the dimensions of an ordinary meter stick with a scale graduated so that the length of the intervals on the scale progresses geometrically. Interval lengths are thus the products of 1.25 and the antilogarithm of 0, 0.05, 0.10, 0.15, 0.20, etc. The value 1.25 is the height of a 0.5 ml. volume of water standing in the glass tubing selected for the columns used. Collection of the samples in geometric progression as described reduces the titration error, particularly in the later samples which would contain increasingly smaller concentrations of fumaric acid per unit volume if linear collections were made. While the collections are proceeding, 1 drop of thymol blue indicator (see "Reagents") is placed in each of the tubes. The first tube which shows indicator discharge marks the beginning of the approximate fumarate position; collections are continued in this way until the effluent amyl alcohol-chloroform solution ceases to change the indicator color to red. The tube before the first tube which shows indicator discharge, the tube after the last tube which shows indicator discharge, and each tube showing indicator discharge are then titrated with 0.004 N sodium hydroxide with a 2 ml. micro burette. The titration is conducted by admitting small amounts of the standard alkali and shaking until the first blue tint appears through the emulsion. Each value (ml. of 0.004 N NaOH) obtained is plotted against the fraction number and the accumulated titrations are calculated to fumaric acid by the formula

$$\text{Mg. fumaric acid} = \Sigma_a^o t \times F$$

where t = ml. of sodium hydroxide required for the fraction, a = the first fraction measured (*i.e.*, the tube before the first tube containing discharged indicator), o = the last fraction measured (*i.e.*, the tube after the last tube which showed indicator discharge), and F = normality of the sodium hydroxide × 58.0.

After three determinations have established a recovery error of less than 5 per cent, the plotted curves are compared. When the mode (peak of the curve) for each curve occurs at the same interval and when the volumes of standard alkali indicated by the mode are equal within a range of 0.03 ml., the procedure may be checked by using a mixture of acids containing fumaric acid (*e.g.*, acetic, fumaric, and malic acids). The position of fumaric acid in the effluent phase is thus determined and the interval or fraction at which fumaric acid first appears, the fraction number at the mode, and the fraction number marking the complete release of fumaric acid from the column are used as guides for subsequent measurements. When either reagents or apparatus are changed, the calibration must be repeated.

Preparation of Sample for Measurement; Deproteinization—Approximately 10 gm. of the minced sample of tissue (or 10 ml. of blood or plasma) are mixed with acetone and the suspension is transferred to a 200 ml. volumetric flask. 1.2 ml. of 10 N sulfuric acid are added and acetone is introduced until the level of the total mixture is within 5 to 10 ml. of the mark. After mixing, the flask is refrigerated overnight. Finally, the volume is adjusted to the mark with acetone and the mixture is filtered through a 15 cm. No. 50 Whatman filter paper. Three 40 ml. aliquots are taken to apparent dryness by placing them under an infra-red lamp and directing a current of air over them, the height of the lamp being adjusted so that the acetone will not boil. Overheating is to be avoided because of the possibility of esterification with the solvent. To the dry residue is added 1 ml. of 2.0 N sodium hydroxide. After the alkali is mixed with the extract, the pH is adjusted to 2 ± 0.5 with dropwise additions of 5 N sulfuric acid, by using indicator paper (Hydrion). Water is then added until the total volume is 2 ml. The acid-acetone procedure does not free the material completely from biuret-positive materials, but any such materials remaining do not appear to interfere with the measurement.

Transfer to Non-Aqueous Phase—The above extract, or an equal volume of aqueous non-protein solutions of fumaric acid, such as urine, adjusted to pH 2 ± 0.5 is mixed intimately with 2 gm. of silica gel (see "Reagents"), suspended in 20 ml. of chloroform, and the suspension is poured into the column. As the chloroform drains through the silica gel, the top of the silica gel column is kept moist with repeated small additions (1 ml. each) of chloroform from a dropping pipette. When the silica gel column settles to an unbroken mass, the chloroform above the top of the silica gel is permitted to disappear into the column and immediately the column is filled with 30 per cent amyl alcohol-chloroform. The first 5 ml. of the liquid issuing from the column are discarded, then the flow is continued until 45 ml. of amyl alcohol-chloroform are collected, the column being refilled to maintain the flow. This amyl alcohol-chloroform mixture containing the fumaric acid is taken to dryness at room temperature by directing moving air over the material and the residue is set aside for measurement.

Measurement of Sample—To the foregoing residue are added 1 to 1.5 ml. of a 10 per cent amyl alcohol-chloroform solution, and after swirling the beaker so that the sides are rinsed with the solvent, it is covered with a watch-glass and allowed to stand for 1 hour. The material in amyl alcohol-chloroform is introduced on the surface of the column, prepared according to the procedure under the preliminary preparation for the analysis, and the beaker is rinsed twice with 0.25 ml. quantities of 10 per cent amyl alcohol-chloroform solution. The procedure for the measurement of fumaric acid then follows that presented under the calibration procedure.

Calculations—Since the above operations give mg. of fumaric acid measured, the concentration of fumaric acid in the sample tested is calculated as follows:

$$C = \frac{\text{mg. fumaric acid measured} \times P \times 100}{W}$$

where C = concentration in mg. per cent of fumaric acid, P = aliquot factor in deproteinization, and W = sample weight.

Interference and Recovery Data

Studies were first undertaken to ascertain whether the organic acids known to occur in animal materials could be adequately separated by means of a silica gel column employing the aqueous alcohol-chloroform system and thus be shown not to interfere with the estimation of fumaric acid. To this end the method for preparation of the sample described above was followed. For the estimation of these acids the entire band of the acid was measured instead of separate fractions as above. Fractions were not collected because "thiamine adsorption tubes" instead of the regular columns were used. The smaller columns permitted measurement of the acids which were released more slowly than fumaric acid in a shorter time. The position of each acid was determined by first examining when and where the acid would occur in the effluent phase. Acids immediately on either side of fumaric acid or coming before fumaric acid were released from the column with lower concentrations of alcohol-chloroform, while later acids required higher concentrations. Positions for malic, lactic, and oxalic acids, with 20 per cent butanol-chloroform, and citric acid, with 35 per cent butanol-chloroform, were similarly determined. The position of succinic acid was determined with 10 per cent butanol-chloroform. The position of fumaric acid was determined with 4, 5, and 10 per cent butanol-chloroform. The distribution coefficient for the acid under consideration would largely determine the phase selected; *i.e.*, acids less soluble in butyl alcohol and more soluble in water would require larger amounts of butyl alcohol than acids whose solubilities with respect to these phases were reversed. When amyl alcohol was substituted for butyl alcohol, the positions for the acids remained unchanged. In a previous report (Marshall, Orten, and Smith (6)) the sequence of acids is given. When acid mixtures were analyzed, the mobile phase was changed while the analysis was in progress by adding the new mobile phase immediately after the effluent acid of the previous phase was released. This addition of new non-aqueous phase followed the removal of any mobile phase, already present above the column, by means of a pipette.

Table I

Recovery of Acids from Aqueous Solutions

The results are expressed in mg.

Acid	Mixture I		Mixture II		Mixture III	
	Acid added	Acid found	Acid added	Acid found	Acid added	Acid found
Fumaric	0.75	0.73	1.50	1.50	2.30	2.47
Succinic	0.25	0.29	0.50	0.46	1.50	1.13
Malic			1.0	1.16	3.10	3.14
Citric					3.40	3.25
Lactic					2.25	2.16
Oxalic					5.51	4.79

With these techniques, pure acid mixtures were analyzed and the results are given in Table I. Additional mixtures were tested for interfer-

TABLE II

Recovery of Fumaric Acid Added to Various Biological Materials

Determination No.	Urine			Plasma						Protein hydrolysate			Liver			Muscle		
				Without deproteinization			With deproteinization											
	Added	Found	Recovery	Added	Found	Recovery	Added	Found	Recovery	Added	Found	Recovery	Added	Found	Recovery	Added	Found	Recovery
	mg.	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*
1	5.8	5.7	98	2.98	3.10	104	1.00	1.02	102	0.14	0.15	107	320	330	103	410	406	99
2	5.8	5.5	95	1.45	1.60	108	1.00	1.03	103	0.28	0.23	82	320	333	104	410	414	101
3	5.8	5.9	102							3.60	3.30	92	320	330	103	410	365	89
4	5.8	5.9	102										1440	1372	98	410	394	96
5	0.66	0.71	107															
6	0.52	0.50	96															
7	0.32	0.40	125															
8	0.23	0.28	122															
9	0.22	0.29	126															
10	0.098	0.096	98															
Average recovery...			107			106			103			94			102			96

ence alone. Pyruvic, glutamic, aspartic, uric, acetic, β-hydroxybutyric, aconitic, tricarballylic, oleic, palmitic, malic, malonic, and creatine represent a partial list of other acids which were found not to interfere with the measurement of fumaric acid.

Studies of the recovery of pure fumaric acid added to various biological fluids and tissues were made (see Table II). Varying quantities of fumaric acid were added to urine, which was found to contain no detectable amount of this acid.

Although it was known that large molecules could interfere with partition in the chromatographic process (Martin and Synge (7)), the attempt was made to analyze some biological materials without prior separation of the contained protein. Whole blood, under these conditions, could not be analyzed because the discharge of acid hematin into the collecting vessels masked the color of the indicator during titration. The data indicate a somewhat high recovery of fumaric acid added to fresh plasma without previous deproteinization. However, satisfactory recoveries of fumaric acid added to plasma and then deproteinized with acid-acetone were obtained.

On the supposition that there might be interference by compounds having peptide linkages, but not necessarily protein, an enzymatic hydrolysate of casein was analyzed after fumarate was added. Satisfactory recoveries were obtained.

Satisfactory recoveries of small amounts of fumaric acid added to liver and muscle were also obtained.

Application to Tissues

To apply the method to the determination of fumarate in tissues under carefully controlled conditions, eighteen normal adult rats of the August by Copenhagen strain[1] were maintained on a stock ration of commercial dog chow. Twelve of these animals having weights of 285 ± 15 gm. were selected and fasted 18 hours. They were then anesthetized by intraperitoneal injections of pentobarbital sodium and after 15 minutes the animals were opened by a ventral abdominal incision and exsanguinated from the aorta. The blood was pooled in a heparinized vessel and the liver, kidney, gastrocnemius muscle, and brain were removed, also pooled, and immediately frozen with dry ice. The pooled tissues were crushed to a fine powder which was weighed while cold, but not frozen. After homogenizing in a Waring blendor with acetone, the material was transferred quantitatively from the blendor into a volumetric flask with acetone and from this point the procedure followed that previously outlined.

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[1] **Appreciation is expressed to Dr. Wilhelmina Dunning of the Department of Pathology for furnishing these rats.**

Blood—When samples of blood having a weight of 4 gm. were chromatographed, titratable acidity was not evident in the interval samples on either side of the interval mean. That there appeared some acidity in Fraction 25 (the mode, as previously described), however, suggested the likelihood that some fumaric acid was present. The use of a 12 gm. sample confirmed this observation; yet the quantity present was not sufficient to acidify every interval. This accounts for the values for blood being expressed as "less than 0.3 mg. per cent." The data for the measurement are shown in Fig. 1.

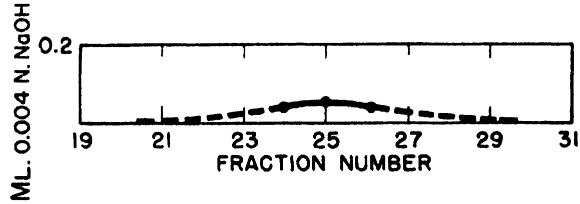

FIG. 1. An estimation of fumaric acid in a 12 gm. sample of blood. The dashed portion of the line indicates that the acidity of these samples was too small to measure. These data indicate that the fumaric acid content of whole blood was less than 0.3 mg.

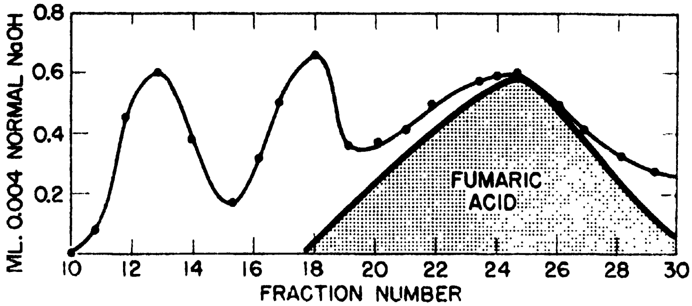

FIG. 2. The results of the first measurement of fumaric acid in brain tissue. The stippled area indicates the concentration of fumaric acid calculated from the titration of Fraction 25 (the mode of the theoretical curve); the value is 14 mg. per cent.

Brain—Brain lipides reduced the rate of flow through the column, samples of brain weighing 5 gm. requiring 18 hours for the chromatographic process. When the sample of brain was smaller, however, the rate of flow approached the normal. The data are shown in Fig. 2. It is evident from the nature of the curves plotted from the titration values that two other acids in addition to fumaric were titrated and that one of these interfered with the determination of fumaric acid. The nature of these two acids was not determined. However, when the effluent material from a similarly treated sample of brain was chromatographed through a second column, the data of Fig. 3 were secured. These data showed a fumaric acid concentration in normal brain of 15 mg. per cent under the conditions of the experiment.

Since repeated measurements of pure fumaric acid added to tissues indicated that total acidity in the logarithmically increasing volumes of the effluent plotted against fraction number described a curve which approximates the normal curve of error, the rechromatographed curve for brain (Fig. 3), therefore, was examined for normality. With the mode at Fraction 25 and Fractions 19 to 31 representing the intervals of a frequency distribution (frequency of hydrogen ions), cumulative frequencies were plotted on probability graph paper. The "fitted" cumulative normal distribution (Wilks (13)), a straight line through the previously plotted cumulative frequencies, gave the dotted area of Fig. 2. This

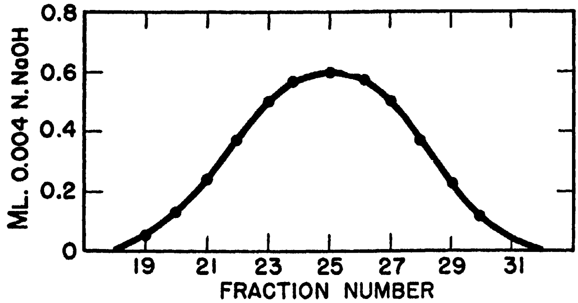

Fig. 3. An aliquot of the brain sample (Fig. 2) after redistribution through a second column. The similarity between this curve and the theoretical curve (Fig. 2) confirms the identity of the fumaric acid and indicates a concentration in the sample of 15 mg. per cent.

Table III

Observed Distribution of Fumarate in Pooled Tissues of Twelve Rats Fasted 18 Hours

Tissue	No. of determinations	Average fumaric acid measured	Standard deviation	Average concentration of fumaric acid, mg. per 100 gm. wet tissue
		γ	γ	
Brain	2	830	*	15.0
Kidney	4	404	65	9.5
Liver	4	172	5.7	7.8
Gastrocnemius muscle	4	127	7.8	2.3
Blood	3	16		<0.3

* Range of measured values, 40 γ.

stippled area thus became a "corrected curve" for the original data which permitted the heavy curve of Fig. 2. This similarity between the observed curve and the theoretical curve thus supported further the value for normal brain.

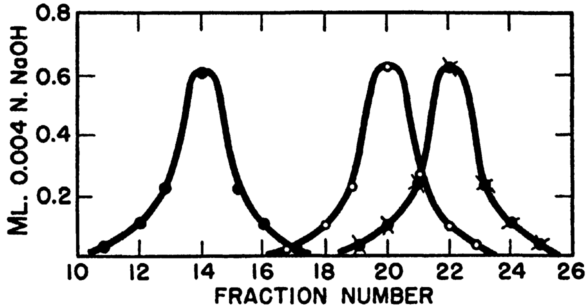

Fig. 4. The shift in mode of the curves showing the concentration of fumaric acid in successive intervals illustrates the influence of change in composition of the mobile phase. ●, 10 per cent amyl alcohol-chloroform; ○, 5 per cent amyl alcohol-chloroform; ✕, 4 per cent amyl alcohol-chloroform.

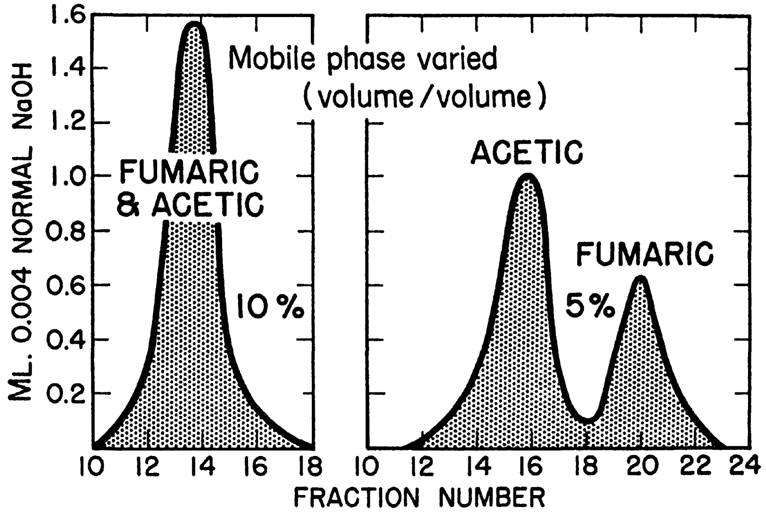

Fig. 5. The separation of fumaric and acetic acids from a mixture by the use of a mobile phase containing a lower concentration of amyl alcohol.

Liver and Muscle—In four samples of liver, in which the average mass of fumaric acid measured was 172 γ, there was represented a concentration in liver of 7.8 mg. per cent. The average weight of fumaric acid measured in four samples of muscle was 127 γ, which corresponded to a concentration of 2.3 mg. per cent. This value is somewhat lower than that reported for pigeon breast muscle by Annau *et al.* (1). However, the method employed by these investigators was undoubtedly less specific than the present one. Satisfactory recovery data for these tissues were secured, as shown in Table II.

Kidney—Because of the large standard deviation for the average value obtained with kidney tissue, Table III, additional evidence was sought concerning the homogeneity of the effluent fumaric acid. In this connection a column made from glass tubing having an internal diameter of 6 mm. was used. The position of fumaric acid was ascertained by employing 10, 5, and 4 per cent amyl alcohol-chloroform. These positions are indicated in Fig. 4. The tendency of this curve displacement to resolve acid mixtures is demonstrated by the effect of the change in composition of the mobile phase on the separation of a mixture of fumaric and acetic acids as shown in Fig. 5. The value for fumaric acid concentration in kidney tissue so resolved (Fig. 6) was 9.5 mg. per 100 gm. of wet tissue.

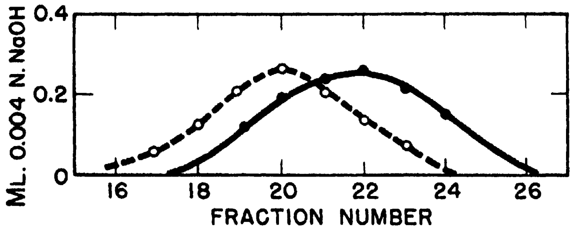

FIG. 6. Graphic representation of the estimation of fumaric acid in kidney tissue. Solid line, 4 per cent amyl alcohol-chloroform; dash line, 5 per cent amyl alcohol-chloroform. This verifies both the identity and the homogeneity of the acid being measured (see Fig. 4).

DISCUSSION

The amounts of fumaric acid found in the various tissues analyzed are surprisingly large and probably reflect the metabolic activity of the tissue, since fumaric acid is believed to be an important intermediate in the "citric acid cycle" and hence in the final common pathway of the metabolism of carbohydrates, fats, and proteins. The significantly high concentration in brain, for example, may be related to the alleged exclusive use of carbohydrate for energy by this tissue. The concentration of fumarate in kidney might suggest that fumarate, like citrate, is the counterpart of urinary ammonia in acid-base mechanisms. The relatively large amount of fumaric acid in the liver is in harmony with the recognized position of this organ in the intermediary metabolism of the major foodstuffs. The low blood fumarate found suggests the lack of mobility of tissue fumarate. These questions, of course, can be answered only by further experimental work. The chromatographic method for fumaric acid just described would appear to be ideally suited for this purpose, since it is possible to determine not only fumaric acid but also, and on the same samples, citric, malic, succinic, and perhaps other organic acids involved in the final oxidative disposition of metabolites.

SUMMARY

A satisfactory method for the determination of small amounts of fumaric acid in biological materials by partition chromatography is described. The procedure can be used to determine as little as 0.05 mg., and is specific. Satisfactory recoveries of small amounts of fumaric acid added to the urine, plasma, casein hydrolysate, liver, and muscle were obtained.

Several tissues of the normal rat were examined for fumarate concentration. Brain showed the highest concentration, with kidney and liver next. The lowest concentration of fumaric acid of the tissues tested was observed in blood.

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THE METABOLISM OF α-ESTRADIOL IN VITRO*

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It has been repeatedly demonstrated that estrogens are inactivated *in vivo*, and from experiments conducted both *in vivo* and *in vitro* it appears that the liver is the major site of estrogen inactivation (1, 2). Although the inactivating mechanism has been studied (3–8), the nature of the metabolites formed *in vitro* has not been ascertained by actual isolation and identification. The present investigation was undertaken with the latter object in mind.

α-Estradiol-17-hemisuccinate was dissolved in a Krebs-phosphate medium buffered at pH 7.4 and incubated with rat liver slices at 37° for 4 hours. The ratio of hormone to liver tissue was approximately 1:500 parts by weight; in all, 845 mg. of α-estradiol (as hemisuccinate) were used. On extraction and fractionation of the incubation mixture, 54 mg. of estrone and 328 mg. of α-estradiol were obtained in crude crystalline form. These products were purified and identified by melting point and mixed melting point determinations with the corresponding authentic specimens; estrone was further identified by carbon and hydrogen analysis of the acetyl derivative. The data on the recovery of the various phenolic fractions are summarized in Table I.

EXPERIMENTAL[1]

Preparation of α-Estradiol-17-hemisuccinate—The procedures followed were similar to those previously described by Pincus and Pearlman (9) for the preparation of this derivative, except that ether-pentane was substituted for aqueous methanol as a solvent for crystallization. Beautiful white needles were obtained, m.p. 165–165.5°.

$C_{22}H_{28}O_5$. Calculated, C 70.94, H 7.58; found, C 70.96, H 7.74

Incubation—A total of 845 mg. of α-estradiol in the form of its 17-hemisuccinate was incubated with male rat liver slices in ten runs. Run 1 is described as a typical experiment. A solution of 84 mg. of α-estradiol (as hemisuccinate) in 1 ml. of ethanol was added dropwise with shaking to 840 ml. of a Krebs solution which had been buffered with phosphate to

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* This work was supported by grants-in-aid from the United States Public Health Service, and from the Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

[1] All melting points reported here are corrected.

pH 7.4 and which contained no glucose. The solution of estrogen was equally distributed in six culture type Roux bottles, and to each bottle were added 7 gm. of liver slices obtained with the aid of a Stadie-Riggs microtome. The bottles after capping with rubber (the gas phase was air[2]) were placed on a mechanical vibrator for 4 hours in an incubator at 37°. The incubated material from Runs 1, 2 to 5, and 6 to 10 were worked up separately by methods described below; pooled incubation mixtures were maintained in a frozen state prior to extraction.

Extraction and Fractionation Procedures—The supernatant fluid was decanted and the liver slices washed with a little water. The aqueous phase was acidified to pH 3 with dilute HCl and gently shaken once with 0.5 volume, and three times with 0.3 volume of butanol. The butanol extracts were neutralized with a drop or two of concentrated NH_4OH and evaporated *in vacuo*. Meanwhile, the liver slices were ground with sand and repeatedly extracted with liberal quantities of boiling methanol. The methanol extracts on cooling to room temperature deposited some insoluble material which was removed prior to evaporating the extracts *in vacuo*. The residues from the butanol and methanol extracts were combined and distributed between water (acidulated to pH 3) and ether; the aqueous phase was further extracted with ether. The ether extracts were combined and back-washed with a little water. The aqueous phase (I) was saved (see below). The ether contained a small quantity of acidic material (II) which was removed prior to evaporation. The residue (neutral substances plus phenols) was distributed between petroleum ether and 90 per cent methanol. The latter phase was brought to dryness and treated with acetone; the acetone-insoluble material was repeatedly taken up in alcohol and precipitated with acetone. The acetone-soluble material thus obtained was taken up in benzene and extracted four times with equal volumes of N NaOH. The alkaline extracts were acidified to Congo red and thoroughly extracted with ether to obtain the "free" phenols. Repeated partitioning of the latter between benzene and 0.3 M Na_2CO_3 (10) yielded strongly acidic and weakly acidic phenols. The latter were separated into ketonic and non-ketonic moieties with the aid of Girard's Reagent T (11). For the weights of the respective phenolic fractions and of the crystallizates obtained therefrom, see Table I.

Ketonic Weakly Acidic Phenols—This fraction was easily induced to crystallize on treatment with aqueous ethanol. The crude crystals (Runs 1 to 10, see Table I) were pooled and further purified from the same solvent to yield 23 mg., m.p. 258–259°. It gave no depression in melting point on admixture with authentic estrone. Acetylation of a specimen of the isolated product yielded crystals, m.p. 123–124°, which gave no depression in melting point on admixture with authentic estrone acetate.

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[2] DeMeio *et al.* (8) observed complete inactivation of α-estradiol by liver slices in oxygen or in air.

$C_{20}H_{24}O_3$. Calculated, C 76.89, H 7.74; found, C 76.75, H 7.76

Non-Ketonic Weakly Acidic Phenols—Crude crystals (Table I) of α-estradiol formed readily from this fraction on treatment with aqueous ethanol. These were pooled, repeatedly crystallized from the same solvent to yield a product, m.p. 174–176°, which did not depress the melting point of authentic α-estradiol on admixture.

The mother liquor from the above was worked up to yield an additional 31 mg. of crude α-estradiol. The 76 mg. of material remaining in the final mother liquor were combined with 113 mg. of non-crystalline material obtained from the benzene phase on partitioning the pooled strongly acidic phenols (Table I) between benzene and 0.2 M Na_2HPO_4.[3] From this fraction (76 + 113 mg.), 44 mg. of digitonin-precipitable material were obtained; it yielded 25 mg. of crude α-estradiol. The non-digitonin-precipitable material was acetylated and chromatographed but none of the eluates could be induced to crystallize.

TABLE I

*Incubation of α-Estradiol; Recovery of "Free" Phenols**

The weights are expressed as mg.

Run No.	α-Estradiol† incubated	Weakly acidic phenols				Strongly acidic phenols
		Total ketones	1st crop crystalline estrone	Total non-ketones	1st crop crystalline α-estradiol	
1	84	18	3	36	27	41
2- 5	339	25	14	147	90	149
6-10	422	37	25	200	155	255
Total.....	845	80	42 ‡	383	272‡	445

* Very little "conjugated" or esterified phenol was contained in extracts of the incubated material; no crystalline estrogens could be isolated from such fractions after hydrolysis.

† Calculated content; the estrogen was incubated as the 17-hemisuccinate.

‡ An additional 12 mg. of crude estrone and 56 mg. of crude α-estradiol were isolated from the respective pooled mother liquors; digitonin was employed to advantage in the latter instance.

Strongly Acidic Phenols—The pooled material (445 mg., Table I) was distributed between benzene and 0.2 M Na_2HPO_4 (12); 113 mg. of phenols remained in the former phase. The benzene-insoluble material was acetylated and chromatographed but no crystalline eluates could be obtained.

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[3] Friedgood *et al.* (12) claim that benzene-0.3 M Na_2CO_3 partitioning is grossly inaccurate for separating estriol from the estrone-estradiol fraction, and recommend instead benzene-Na_2HPO_4 partitioning.

Other Fractions—The following fractions were also examined in the search for estrogen metabolites but without success: the "free" neutral substances (90 per cent methanol fraction after partitioning with petroleum ether), the phenols from the alkaline hydrolysate of the "free" acids[4] (II), and the phenols from the acid hydrolysate of the aqueous phase (I) (see above); the weights of these fractions were respectively 67, 189, and 56 mg.

DISCUSSION

These *in vitro* experiments, which are based on the actual isolation of metabolic products, indicate that α-estradiol can be converted into estrone, a process which is also known (1, 2) to occur *in vivo*. That estrone is not the sole metabolite may be inferred from the fact that only about half of the estrogenic substance incubated could be recovered as biologically active material.[5] Other products such as β-estradiol and estriol, which are formed *in vivo* (1, 2) from either estrone or α-estradiol, were sought for in the present study but without success.[6] It is noteworthy, however, that the strongly acidic phenolic fraction was comparatively large (see Table I); this fraction might have been expected to contain estriol. Efforts to cast light on the nature of the biologically inactive metabolites of estrogenic hormones, a subject of much conjecture, proved fruitless: the fractions examined were the neutral substances and acids.

SUMMARY

Following incubation of α-estradiol-17-hemisuccinate with surviving rat liver slices, a small amount of estrone (6 per cent of the original α-estradiol content) and α-estradiol (about 40 per cent) were isolated. As much as 50 per cent of the incubated estrogen thus remains unaccounted for.

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[4] Any α-estradiol hemisuccinate failing to undergo hydrolysis during incubation would have appeared in this fraction. The fact that no α-estradiol could be isolated from this fraction following alkaline hydrolysis indicates that *in vitro* hydrolysis of the ester was practically complete. By way of comparison, it is noteworthy that Schneider and Mason (13) observed a rapid hydrolysis of dehydroisoandrosterone hemisuccinate when incubated with liver slices.

[5] In earlier experiments by DeMeio *et al.* (8), wherein the biological inactivation of hormone was complete, the ratio of α-estradiol to liver tissue was 1:5000 parts by weight.

[6] An explanation might be sought in the well recognized rôle that species differences play in estrogen metabolism.

The technical assistance of Miss Emily Cerceo and Miss Ann Hughes is gratefully acknowledged. Mr. James Rigas performed the microanalyses. We are indebted to the Ciba Pharmaceutical Products, Inc., Summit, New Jersey, and to Roche-Organon, Inc., Nutley, New Jersey, for generous gifts of α-estradiol.

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A CHEMICAL METHOD FOR THE DETERMINATION OF MANNOSIDOSTREPTOMYCIN

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Several analytical methods have been suggested for the determination of mannosidostreptomycin (streptomycin B) (5, 6). Titus and Fried (6, 12) and Plaut and McCormack,[1] who have utilized the quite specific and quite accurate but yet laborious counter-current distribution methods (previously described by Craig) for the isolation and determination of streptomycin B, have noted a number of difficulties that are encountered under certain experimental conditions. Another method is that described by Schenck *et al.* (10) who have combined the maltol test (11) with the microbiological assay. Inasmuch as streptomycin B shows lower antibiotic activity against many microorganisms than does streptomycin A (6, 9), they assume that it is possible to apply simultaneous equations to the results obtained with these assay methods and thus determine the relative quantities of streptomycins A and B present in the preparations under examination. This is theoretically sound if all the antibiotic activity is derived from the streptomycins A and B present, and if streptomycin A and streptomycin B potencies are additive in antibiotic effect. However, this analytical system inherits the difficulties present in both of the methods, including the effect of miscellaneous materials such as metallic ions and carbohydrates on the maltol assay and on the bioassay (2). Also, the bioassay requires a rather long incubation period.

Consideration of chemical methods adaptable to the determination of streptomycin B suggested that the determination of the mannose moiety might serve satisfactorily. This determination could be combined with the maltol analytical method (by which total streptomycin is measured), and the quantity of streptomycin A present in the sample determined by difference. The carbazole test has been used for the identification and estimation of mannose and other reducing sugars by several investigators (1, 3, 7, 8) and offered promise as a quantitative method (7).[2]

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[1] Plaut, G., and McCormack, R. B., unpublished work.

[2] Several months after the procedure here was devised and while this manuscript was in preparation, a preliminary announcement by Emery and Walker (4) indicated that the anthrone reagent was also adapted for use in determination of the mannose moiety of streptomycin B. Preliminary observations in this laboratory have in-

Analytical Method

After some preliminary study the following analytical procedure, which is similar to that of Gurin and Hood (8), was adopted and found to give satisfactory results. A sample containing 0.05 to 0.15 mg. of streptomycin B hydrochloride (equivalent to 0.004 to 0.131 mg. of free base) is diluted to 2 ml. with distilled water in a standardized colorimeter tube and chilled in an ice bath. 5 ml. of concentrated sulfuric acid are added dropwise with shaking to prevent rise in temperature of the solution. Exactly 0.50 ml. of a carbazole solution (0.5 gm. of carbazole, commercial grade, dissolved in 100 ml. of 95 per cent ethanol) is added to each tube, and the contents of the tube are well mixed. When all of the samples and standard solutions of streptomycin A and streptomycin B have been so treated, the group of colorimeter tubes contained in a rack is placed in a vigorously boiling water bath for a 10 minute heating period. At the end of this interval, the tubes are removed from the bath, cooled to room temperature, and compared in a photoelectric colorimeter at two wave-lengths with a distilled water blank to which have been added the carbazole and sulfuric acid reagents. With the Evelyn photoelectric colorimeter, the 540 and 660 mμ filters have been used.

In the tubes containing only streptomycin B, a purple-red color is formed under these conditions, while in tubes containing only streptomycin A the color formed is a yellow-brown. Intermediate colors are found in tubes containing mixtures. The colored complex formed with streptomycin B gives an absorption maximum between 520 and 560 mμ, and the colored complex formed with streptomycin A under these conditions also absorbs in this region. Since absorption by the streptomycin A complex is significant, corrections must be made for the presence of streptomycin A in the samples. At 660 mμ the absorption of the two complexes is practically identical, and the quantity of streptomycin B present in the samples is therefore calculated by application of the equation

$$B = \frac{\left(D_{540}\left(\frac{C_{A_1}}{C_A}\right) - D_{660}\right)}{\left(C_B\left(\frac{C_{A_1}}{C_A}\right) - C_{B_1}\right)}$$

where B is the quantity of streptomycin B in the sample expressed in mg., D_{660} and D_{540} the extinction coefficients as determined in the photoelectric colorimeter,[3] C_A and C_B are the Beer's law constants at 540 mμ per mg.

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dicated that, as for the carbazole reagent used in the method described here, the anthrone reagent is not quite specific for streptomycin B, and correction must be made for the presence of streptomycin A.

[3] If the 0 to 100 scale is used, as in the Evelyn instrument, D can be calculated from a 2 − log G table.

of streptomycins A and B respectively, and C_{A_1} and C_{B_1} are the constants per mg. of streptomycins A and B at 660 mμ. This method of calculation depends, of course, on the applicability of Beer's law, which was confirmed by experiment. Inasmuch as the constants vary slightly from one group of analyses to another, standard samples of streptomycin A and streptomycin B are included with each, and constants are separately determined for each group. Two or three replicates of two dilutions of each sample and standard are included in each group. The analysis is completed by calculating first the Beer's law constants for each group from the extinction coefficients determined with the standard streptomycin A and B preparations. Per mg. of the pure hydrochloride, the range in C_{A_1}/C_A observed has been 0.45 to 0.88; that of C_B, 3.1 to 4.8; and that of C_{B_1}, 0.80 to 1.30.

DISCUSSION

The absorption spectra of the complexes formed by streptomycin A, streptomycin B, mannose, and streptobiosamine[4] with this reagent under these conditions have been studied and the measurements (as compared with the blank cell containing the carbazole and sulfuric acid) are summarized in Fig. 1.

The procedure has been used in the analysis of many mixtures of streptomycin A and streptomycin B, and typical results are presented in Tables I and II. It will be noted that in some cases the recovery of streptomycin B is slightly high. Analysis of unknown mixtures presents a slightly different problem, as a number of possible interfering materials may be present. The analyses presented in Table II are typical for unknown mixtures of streptomycin A, streptomycin B, and inert inorganic material. Analysis of Sample B by the counter-current distribution method indicated the presence of 0.39 mg. of streptomycin B per mg. of sample, and 0.55 mg. per mg. of sample for Sample C. All streptomycin preparations used in these experiments have been hydrochloride salts, and the analyses are stated in terms of these salts. Experiments indicate that other salts (*e.g.* calcium chloride double salt, sulfate) may also be used with correction for the differences in molecular weights.

A number of inorganic and organic substances will interfere with the determination of streptomycin B by this method. As pointed out by Dische (1), Gurin and Hood (7), and Egami (3), many reducing sugars react similarly to mannose. Nearly all carbohydrates tested formed complexes with strong maxima at 540 mμ. Of course, the determination by this method of streptomycin B in samples containing reducing sugar is not feasible, as is shown in Table III. Such substances as amino acids, glycerol, glucosaminic acid, chondrosaminic acid, glucosamine, and *N*-methyl-L-glucosamine do not interfere with this test, and recovery of streptomycin B in the presence of these substances was satisfactory. It was noted that the yellow color formed by samples of octyl alcohol and pentasol interfered with the determination of streptomycin B. As indicated in

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[4] We are indebted to Dr. J. Fried for this material which was free of streptomycin A.

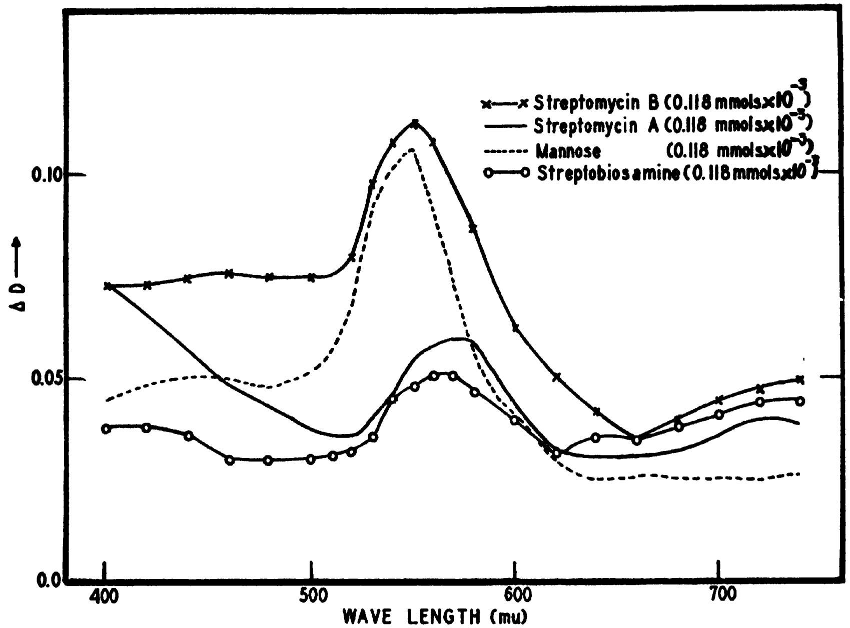

FIG. 1. Absorption spectra of reaction product formed between carbazole reagent and streptomycins and related compounds.

TABLE I

Analyses of Known Mixtures of Streptomycins A and B

Composition of mixture	Streptomycin B found	Recovery
	mg.	*per cent*
0.05 mg. Streptomycin B 0.10 " " A	0.047	94
0.10 " " B 0.10 " " A	0.104	104
0.15 " " B 0.10 " " A	0.142	95
0.05 " " B 0.20 " " A	0.053	106
0.05 " " B 0.30 " " A	0.052	104

Table IV, certain inorganic ions such as iron, copper, cobalt, and chromium also interfere with the determination of streptomycin B. In most cases, the quantities of these inorganic ions necessary to result in colored solutions are greater than those expected in samples susceptible to analysis by this method.

Extensive use of the analytical procedure has indicated that the tubes should not be exposed to sunlight. Such exposure results in the carbazole-sulfuric acid solution becoming a rather deep green, and leads to rather unsatisfactory determinations. Care must also be taken in the addition of the sulfuric acid to the solution containing the streptomycin. If the temperature of the solution rises during the addition of the acid, the results obtained with replicate tubes are often quite variable, and the colors formed with the carbazole may be quite different from those usually observed.

TABLE II

Analyses of Unknown Mixtures for Streptomycin B

Sample	Size of sample	Streptomycin B found	Calculated streptomycin B, mg. streptomycin B per mg. sample	Streptomycin B determined by counter-current extraction,[*] mg. streptomycin B per mg. sample
	mg.	*mg.*		
A	0.050	0.037	0.74	
"	0.10	0.071	0.71	
"	0.20	0.148	0.74	
B	0.050	0.021	0.42	0.39
"	0.10	0.038	0.38	0.39
"	0.20	0.079	0.39	0.39
C	0.10	0.057	0.57	0.55
"	0.20	0.118	0.59	0.55

[*] We are indebted to Mr. F. Russo-Alesi for these analyses by the Plaut-McCormack method (unpublished work).

The use of a blank tube for comparing the color intensity obtained with known and unknown samples has resulted in simplifying the standardization procedure often needed in photometric determinations, and perhaps explains the variation observed in the constants. For example, experimental observations have indicated that the heating period is not critical and that the color development is almost maximal within 5 minutes after immersing the tubes in a boiling water bath. A heating period of 40 minutes resulted in slightly more intense coloring, but the blank tube was equally affected and the determinations were therefore unaffected. Also, the interval between color development and measurement is not critical. Measurements taken over a 24 hour period indicated that, after an interval of 2 hours, the colors began to darken; but the blank tube darkened in the same degree, and the accuracy of the determinations was unaffected.

The intensity of the color formed evidently depends upon two steps in the procedure. Perhaps the most important is the final concentration of the sulfuric acid. The changes in Beer's constant at 540 and 660 mμ for streptomycin A and streptomycin B with change in concentration of sulfuric acid are shown in Table V. When the concentration of acid was reduced below 20 N, the carbazole precipitated from solution and resulted in a turbid mixture; at 18 N, no color was obtained. It is evident

TABLE III

Effect of Various Organic Substances on Analyses for Streptomycin B

Substance	Quantity added to streptomycin B sample, mg. substance per mg. streptomycin B (range tested)	Recovery of streptomycin B, average
		per cent
α-Alanine	2 - 6	103
L-Arginine·HCl	2 - 6	101
L-Cystine	2 - 6	101
DL-Isoleucine	2 - 6	104
L-Proline	2 - 6	101
L-Tryptophan	2 - 6	101
L-Glucosamine·HCl	2 - 6	100
Chondrosaminic acid	2 - 6	103
Glucosaminic acid	2 - 6	97
N-Methyl-L-glucosamine	2 - 6	101
Streptobiosamine	2 - 6	97
Glycerol	2 - 6	101
Mannitol	2 - 6	101
Octyl alcohol (technical)	6 -20	Indeterminate
Pentasol (technical)	6 -20	"
L-Arabinose	0.2- 0.66	"
D-Fructose	0.2- 0.66	"
D-Galactose	0.2- 0.66	"
Lactose	0.2- 0.66	"
D-Mannose	0.2- 0.66	"
Sucrose	0.2- 0.66	"
Starch	0.2- 0.66	"
D-Xylose	0.2- 0.66	"

TABLE IV

Effect of Various Inorganic Ions on Analyses for Streptomycin B

Ion	Quantity added to sample	Recovery of streptomycin B
	mg.	*per cent*
Aluminum	0.10	102
Calcium	1.0	104
Chromium	0.10	Indeterminate
	0.01	"
	0.001	83
	0.0005	96
Cobalt	0.10	Indeterminate
	0.01	"
	0.001	95
Copper	0.10	Indeterminate
	0.01	"
	0.001	78
	0.0005	98
Iron	0.10	Indeterminate
	0.01	99
	0.001	103
Manganese	1.0	98
Potassium	10.0	101
Sodium	10.0	103
Zinc	1.0	98

TABLE V

Effect of Sulfuric Acid Concentration on Beer's Constant

Concentration of sulfuric acid	Beer's constant			
	Streptomycin A		Streptomycin B	
	540 mμ	660 mμ	540 mμ	660 mμ
N				
33.4	0.25	0.11	0.72	0.15
32.8	0.27	0.20	0.92	0.21
31.0	0.33	0.24	0.95	0.30
29.5	0.46	0.28	1.75	0.35
25.7	0.93	0.50	3.87	1.18
25.0	1.02	0.72	3.94	1.68
22.8	1.38	0.85	4.14	1.80
21.5	1.36	0.85	3.63	0.99

that, if the final concentration is either too high or too low, the sensitivity of the test is reduced.

The color intensity also depends in part on the quantity of carbazole added. It is very important that each tube receive the same quantity of carbazole, and that the contents of the tube are well mixed before heating in the water bath. In a number of experiments, the quantity of carbazole was varied from the 0.50 ml. suggested. Visually the colors were slightly changed, but the blank tube also changed equally, and the colorimeter measurements were unaffected. In the above procedure an excess of carbazole is added, and preliminary experiments indicate that as little as 0.05 ml. of a 0.1 per cent solution might suffice.

This analytical procedure as described has been used successfully for the analysis of rather pure preparations of streptomycins. Application to such preparations as fermentation media, blood and body fluids, and other materials would probably depend on the elimination of interfering materials such as carbohydrates and metallic ions. It is possible that clarification procedures similar to that mentioned by Schenck *et al.* (10) for the concentration and extraction of streptomycins from fermentation broth would be sufficient.

SUMMARY

A chemical method for the determination of mannosidostreptomycin (streptomycin B) in purified preparations based on the color formed with carbazole in sulfuric acid solution has been described.

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STUDIES ON THE GROWTH INHIBITOR FRACTION OF LIMA BEANS AND ISOLATION OF A CRYSTALLINE HEAT-STABLE TRYPSIN INHIBITOR*

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Ham and Sandstedt (2) and, subsequently, others have shown that there is a trypsin inhibitor in soy beans and other legumes which inhibits the growth of certain animals. At first it was believed that the components of beans which alter the nutritive value of proteins exert growth inhibition by their ability to inhibit proteolysis in the gastrointestinal tract. Recently Klose and associates (3) have demonstrated that the growth-inhibitory fraction of Lima beans affects the growth of rats not by its action on proteolysis but by some other mechanism not yet identified. This conclusion was drawn because a diet including hydrolyzed proteins also resulted in a decrease of growth when supplemented with the growth-inhibitory fraction. More recently Westfall and coworkers (4) came to similar conclusions after investigating the crude trypsin inhibitor fraction of soy beans.

The aim of the present report is to confirm the observations of the earlier investigators concerning the growth-inhibitory effect of the crude Lima bean fraction and to describe the isolation from this fraction of a crystalline, heat-stable globulin which powerfully inhibits trypsin and moderately inhibits chymotrypsin. Swiss mice, which were employed in the feeding experiments, were found to be extremely sensitive to the growth-inhibitory fraction of Lima beans.

EXPERIMENTAL

Preparation of Crude Trypsin Inhibitor Fraction of Lima Beans—The crude inhibitor, which appears to contain at least two proteinase inhibitors, was prepared by the following modification of Kunitz' method (5) for the initial purification of the soy bean trypsin inhibitor. 2 kilos of ground chloroform-defatted Lima beans were extracted with 10 liters of distilled water containing 70 cc. of concentrated sulfuric acid at 20–25° for 1 hour with occasional stirring. The inhibitor proteins were adsorbed from the filtrate on a mixture containing 50 gm. of bentonite and 50 gm. of Hyflo for a period of 10 minutes, during which time the mixture was occasionally stirred. It was then centrifuged. The solid portion was mixed with 1200 cc. of distilled water and again centrifuged. The supernatant and washings were discarded. The trypsin inhibitors were eluted with 540 cc. of water to which 60 cc. of pyridine were added. The mixture was warmed to 25°, shaken for 10 minutes, and then centrifuged. The solid portion was washed with 200 cc. of 5 per cent pyridine. The combined solutions were dialyzed for 48 hours through cellophane membranes in order to remove the pyridine. To the pyridine-free solution 400 gm. of ammonium sulfate were added in order to precipitate the inhibitor fraction. The precipitate was dissolved in 100 cc. of distilled water and dialyzed until free of ammonium ions. This required about 24 hours. The resultant solution was then lyophilized or precipitated with 3 volumes of acetone and dried *in vacuo* over P_2O_5. On the addition of acetone, a colloidal suspension sometimes formed. Immediate precipitation occurred on the addition of about 100 mg. of sodium chloride to the suspension. The yield, varying somewhat with each batch of beans used, was about 7.5 gm. of light yellow powder. In the experiments to be described, this preparation will be designated as "crude trypsin inhibitor."

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* A preliminary report of this work was presented before the American Society of Biological Chemists at the meeting of the Federation of American Societies for Experimental Biology at Detroit, April 18–22, 1949 (1).

Isolation of Crystalline Heat-Stable Lima Bean Trypsin Inhibitor—5 gm. of the crude trypsin inhibitor fraction (acetone powder or lyophilized powder) were dissolved in 250 cc. of 1 per cent sodium chloride solution at 35°. 1 gm. of bentonite and 1 gm. of Hyflo were mixed together and added in small portions to the inhibitor solution. The mixture was shaken several times during a period of 10 minutes at room temperature, then filtered through a Büchner funnel. To the clear filtrate, 50 cc. of acetone were added in small portions and the solution was placed in the refrigerator at 5–6° overnight. Microscopic, colorless octahedral crystals formed (Figs. 1 and 2). These were collected in a cooled centrifuge. Crystallization could be initiated by dialyzing against ethyl alcohol for about 30 minutes or by seeding with crystals previously obtained. The crystals were recrystallized three times by being dissolved in 8 cc. of 1 per cent sodium chloride solution followed by the addition, drop by drop, of 2 cc. of acetone. Sometimes a small amount of amorphous or globular material precipitated as soon as the solution became chilled. These noncrystalline precipitates were removed by centrifugation and discarded. The resultant supernatants were then water-clear and yielded crystals much more readily. Sometimes crystallization took place within 1 hour. The crystals were washed with 10 cc. of cooled acetone and dried *in vacuo* over P_2O_5. The yield was 85 mg. of white powder. Another equal quantity of crystals was obtained by placing the first supernatant in the refrigerator at 5–6° for 1 week. An inhibitor fraction was also obtained in the form of microscopic plates from a 1 per cent sodium chloride solution containing 20 per cent ethyl alcohol. This material, however, was

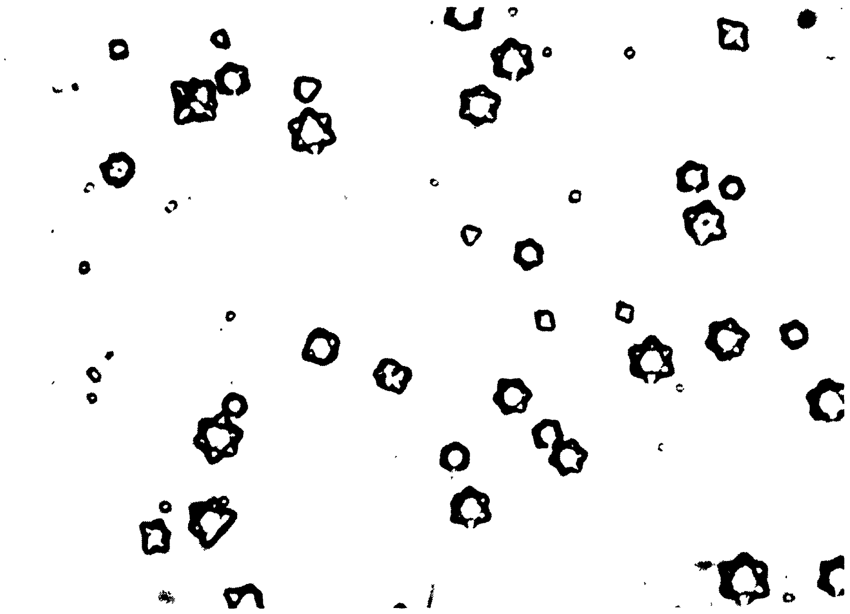

FIG. 1. Crystals of Lima bean trypsin inhibitor, 200 ×

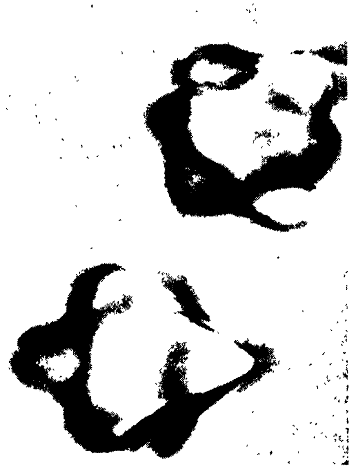

FIG. 2. Crystals of Lima bean trypsin inhibitor, 1000 ×

less active than the crystals obtained from the sodium chloride solution containing 20 per cent acetone.

Properties of Crystalline Lima Bean Trypsin Inhibitor—The activity of the crystals does not change on repeated recrystallizations. There remains, however, a trace of polysaccharide which is not removed by the described procedure. The 4 times crystallized material is a globulin. It is soluble in a 1 per cent sodium chloride solution, and slightly soluble in water. The composition of crystalline Lima bean trypsin inhibitor is that of a typical protein (Table I). It is precipitated by the protein precipitants, acetone, ethyl alcohol, and trichloroacetic acid. It gives the usual protein test and a very strong tryptophan test. It does not dialyze through cellophane. The substance is a powerful inhibitor of crystalline trypsin (Table II). Proteolysis and milk clotting by chymotrypsin are moderately affected (Table III). Proteolysis and milk clotting by pepsin and the action of cathepsin (calf kidney extract in 50 per cent glycerol-citrate solution of pH 4.0) on hemoglobin at pH 4.0 are not affected.

TABLE I

Composition of Crystalline Lima Bean Trypsin Inhibitor in Per Cent Dry Weight

C	H	N	S	P
52.11	6.96	15.80	0.93	0.00

Calculated on an ash-free basis. The ash content was 2.3 per cent.

TABLE II

Casein Digestion by Crystalline Trypsin and Crystalline Chymotrypsin in Presence of Crystalline Lima Bean Trypsin Inhibitor

Experiment No.	Crystalline enzyme		Crystalline Lima bean inhibitor	Inhibition
		mg.	*mg.*	*per cent*
1	Trypsin	0.05	0.02	22
2	"	0.05	0.05	60
3	"	0.05	0.10	100
4	"	0.05	0.20	100
5	Chymotrypsin	0.2	1.00	52
6*	Trypsin	0.05	0.20	100
7*	"	0.05	0.05	60

The quantities of enzymes as given in this table were dissolved in 2 cc. of distilled water. The inhibitor was dissolved in 1 cc. of 1 per cent sodium chloride. To the enzyme-inhibitor mixture, 3 cc. of casein solution of pH 7.6 were added.

* Here the inhibitor was autoclaved at 15 pounds pressure for 5 minutes.

The crystalline Lima bean trypsin inhibitor is exceptionally heat-stable. Autoclaving for 5 minutes at 15 pounds pressure does not affect its activity (Table II). In contrast, the crystalline trypsin inhibitor which

Kunitz (5) isolated from soy beans is completely destroyed on short atmospheric boiling. It was found that crude Lima bean trypsin inhibitor employed in the feeding experiments described below had to be autoclaved for 1½ hours at 18 pounds pressure in order to destroy all of its inhibitory action. Autoclaving for 1 hour at 15 pounds pressure destroyed only 40 per cent of its inhibitory power. The crude acetone precipitate was twice as active in the proteolytic test as the 3 times crystallized trypsin inhibitor; and in the milk-clotting test, the crude inhibitor was about 5 times as active (Table III). This observation leads to the belief that there are at least two proteinase inhibitors present in the Lima bean.

TABLE III

Inhibition of Milk-Clotting Action of Chymotrypsin by Crystalline Lima Bean Trypsin Inhibitor

Experiment No.	Type of inhibitor	Total clotting time	Delay of milk clotting
		min.	*min.*
1	None	4	
2	Crude Lima bean inhibitor	32	28
3	" " " " boiled 15 min.	30	26
4	Crystalline Lima bean inhibitor	10	6
5	" " " " boiled 15 min.	10	6
6	" soy " "	8	4
7	" " " " " " "	4	0

In each of these experiments 1.25 mg. of chymotrypsin were contained in 0.5 cc. of distilled water. 5 mg. of the respective inhibitor were dissolved in 0.5 cc. of 1 per cent NaCl. The crystalline soy bean trypsin inhibitor was kindly furnished by Dr. M. Kunitz of The Rockefeller Institute for Medical Research.

TABLE IV

Growth Inhibition of Mice As Produced by Lima Bean Crude Trypsin Inhibitor

Group No.	Type of feed	Average weight gain in 10 days
		gm.
1	Rockland diet + 10% water (original weight)	7.2
2	" " + 1.7% inhibitor in 10% water autoclaved for 1½ hrs. at 18 pounds pressure	8.2
3	Rockland diet + 1.7% unautoclaved inhibitor in 10% water	0.4

For the estimation of tryptic activity the Folin-Ciocalteu phenol reagent as described by Northrop and associates (6) was employed. Color intensities were measured with the aid of a Cenco-Sheard-Sanford photelometer. The milk-clotting power of chymotrypsin (Table III) was determined at 26° with 5 cc. of a strongly buffered milk of pH 5.0 (7).

Growth Inhibition Produced in Mice by Crude Lima Bean Protein Fraction—Table IV represents a typical series of experiments showing the effect of the crude inhibitor on young mice. Three groups of ten mice, average initial weight 6.8 gm., were used. The Rockland mouse diet used as feed was ground in a grain mill. The inhibitor was suspended in a volume of water representing 10 per cent by weight of the feed employed. The ground feed and the inhibitor suspension were mixed and pressed into cubes by the use of a Carver hydraulic press, 16,000 pounds of pressure per sq. in. being applied.

It may be seen from Table IV that Group 3, receiving 1.7 per cent of the unautoclaved inhibitor, gained only a negligible amount of weight in 10 days, whereas Group 2, receiving the extensively autoclaved inhibitor, showed an average gain of 8.2 gm. Group 1, receiving the Rockland diet without the inhibitor, showed an average gain of 7.2 gm. Food consumption in Group 3 was slightly less than in Groups 1 and 2. When only 1 per cent crude inhibitor was added to the Rockland diet, the differences among the three groups were much less pronounced.

There was not enough of the crystalline Lima bean trypsin inhibitor produced for further feeding experiments. It should be noted that soy beans contain three trypsin inhibitors, one of which has no growth inhibitor activity (8).

SUMMARY

A crystalline trypsin inhibitor of globulin nature has been isolated from the growth-inhibitory fraction of Lima beans. Proteolysis by trypsin is powerfully inhibited, and proteolysis and milk clotting by chymotrypsin moderately so. The proteolytic action of cathepsin and the milk-clotting power and proteolytic action of pepsin are not inhibited.

This trypsin inhibitor somewhat resembles Kunitz' crystalline soy bean trypsin inhibitor. In contrast to his inhibitor, however, the crystalline Lima bean inhibitor is exceptionally heat-stable.

Feeding experiments on Swiss mice have shown that their growth is radically interfered with by supplementing the diet with 1.7 per cent of the crude Lima bean trypsin inhibitor. An extensively autoclaved inhibitor does not inhibit the growth of mice or the activity of trypsin.

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THE EFFECT OF CATHODE RAYS PRODUCED AT 3000 KILOVOLTS ON NIACIN TAGGED WITH C^{14}

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It was reported previously by Proctor and Goldblith (1) that niacin in aqueous solution is relatively stable when subjected to high voltage x-ray and cathode ray irradiations, although some of the niacin is destroyed. No evidence was presented, however, as to the structural changes that occurred in the niacin.

Observations by Sheppard and Burton (2) that fatty acids are decarboxylated by α-rays suggested that a similar change might occur on the irradiation of niacin by x-rays and cathode rays. Although α-rays are classified in the heavy particle group, the ions in this group are fast moving, charged particles which produce electronic excitation and ionization in the material they traverse. In this respect, they are similar to such light particle radiations as x-rays and cathode rays.

In order to investigate the possibility that the destruction of niacin is due, at least in part, to decarboxylation, experiments were conducted in which niacin labeled with C^{14} in the carboxyl group was irradiated with cathode rays which were produced at 3000 kv.

Materials and Equipment

Niacin—Samples of niacin labeled with C^{14} in the carboxyl group were prepared at the Los Alamos Scientific Laboratory and shipped to the Massachusetts Institute of Technology for irradiation. An aqueous solution of niacin (concentration 100 γ per ml.) was prepared for irradiation. 3 ml. samples of this solution were introduced into glass vials (18 mm. in diameter × 65 mm. in length) and the vials sealed for irradiation.

In the investigation reported previously by the authors (1), a stock solution of niacin in absolute ethanol instead of water was used. Dilutions as needed were made with distilled water. The only difference in the effects of cathode ray irradiations on aqueous and alcohol solutions of a substance is in the penetration of the bombarding electrons through the samples. This is illustrated by the equation

$$R_{\text{max.}} = \frac{4.7V}{d}$$

where $R_{max.}$ is the maximum range (mm.) of the electrons into the material, V is the impressed voltage in megavolts, and d is the density of the material expressed in gm. per ml.

For water, $R_{max.}$ is equivalent to 1.41 cm. and for alcohol, 1.79 cm. As the samples used in this study were less than 1 cm. thick, this difference in penetration of aqueous and alcohol solutions played no rôle.

Previous experiments have shown that in the destruction of niacin by cathode ray irradiation it makes no difference whether alcohol or water is used as the solvent for niacin, provided the depth of the solution is within the maximum range of the electrons, which was the case in the experiments here reported. This is substantiated by the values in Table I of this paper and in Table 8 of the previous report (1).

TABLE I

Effect of Cathode Rays Produced at 3000 Kv. on Niacin Labeled with C^{14} in Carboxyl Group As Determined by Chemical Assay

Sample No.	Dosage	Retention
	rep	*per cent*
3	0.66×10^6	94
4	1.32×10^6	79
5	1.98×10^6	55
6	2.64×10^6	45

Irradiation Source—Cathode rays (electrons) were produced by a Trump generator operating at 3000 kv. This generator, which has been described in detail previously (3, 4), operates on the Van de Graaff electrostatic principle and is capable of producing intense sources of pure electrons.

Methods of Assay

Chemical—Niacin was determined chemically by the cyanogen-bromide colorimetric method of Mueller and Fox (5), which was modified to the extent that a solution of 20 per cent ammonium hydroxide was used instead of an ammonia buffer. The standard curve of the niacin, cyanogen bromide, and ammonia reaction, which is a straight line, was checked daily with a sample containing 10 γ of niacin per ml.

Waisman and Elvehjem (6) have emphasized that "the chemical methods now available for the determination of nicotinic acid depend upon the pyridine ring structure." They state, furthermore, that vitamin B_6, which has a pyridine ring structure with a methyl group in the α position, does not react with cyanogen bromide, that none of the amino acids react, and that picolinic acid, α-picoline, trigonelline, and methylpyridinium chloride do

TABLE II

Effect of Cathode Rays Produced at 3000 Kv. on Niacin Labeled with C^{14} in Carboxyl Group As Determined by Radioactivity Measurements of Both Gaseous and Liquid Phases

Sample No.	Dosage	Total counts per sec. of CO_2	Total counts per sec. in solution	Per cent of counts left in solution
	rep			
1	0.17×10^6	1145	11,000	72.4
2	0.33×10^6	2100	11,350	74.6
3	0.66×10^6	2140	7,950	52.2
4	1.32×10^6	4970	6,450	42.4
5	1.98×10^6	6410	4,920	32.4
7	5.28×10^6	8400	3,780	24.9
8	Control	104	15,200	99.3

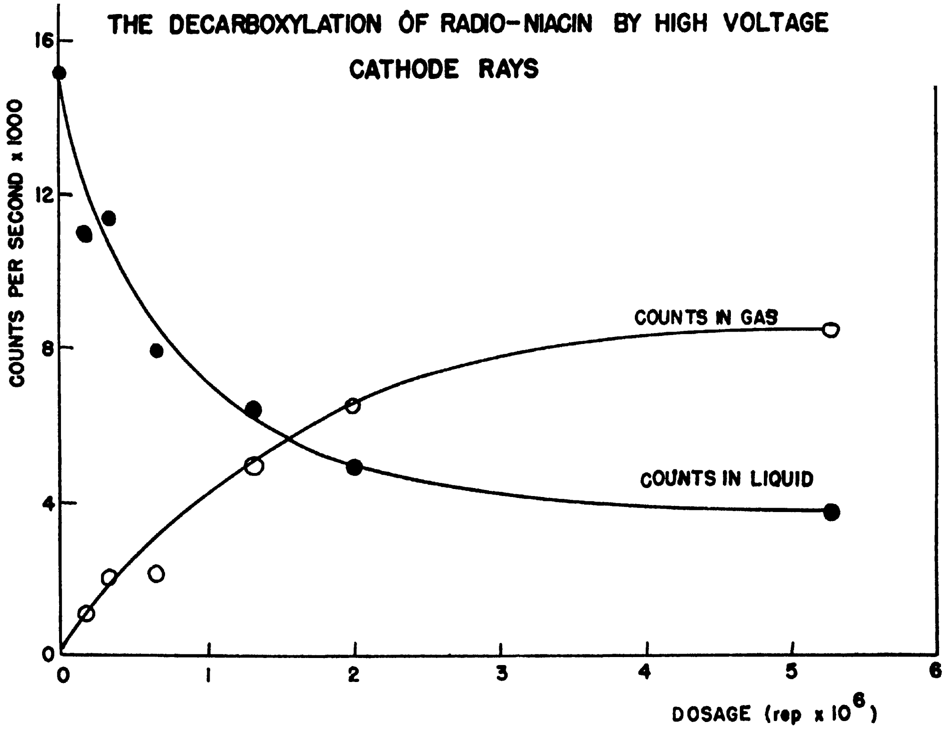

FIG. 1

not react. As the experiments here to be reported dealt with pure solutions of niacin, compounds such as β-picoline, α-aminopyridine, and nepecotic acid, which in high amounts interfere with the cyanogen bromide reaction, did not enter into the picture. Hence the reaction as determined by the cyanogen bromide method was highly specific for niacin.

Radioactivity Assay—The irradiated and control samples, in the ampuls, were each placed in a closed system containing a measured amount of sodium carbonate which was used as a carrier. The ampuls were then broken, and a dilute solution of sulfuric acid was added to the closed system to liberate carbon dioxide. The carbon dioxide was swept into a 10 per cent solution of sodium hydroxide and precipitated as barium carbonate. The barium carbonate was assayed for radioactivity, with the use of a helium-alcohol-filled Geiger-Müller tube having a thin mica window (1.5 mg. per $cm.^2$). Absorption corrections were made according to the method of Yankwich *et al.* (7). Radioactivity remaining in the niacin solution was determined by plating the solution directly onto copper disks (8) and counting as described above.

EXPERIMENTAL

3 ml. samples of tagged niacin solution, in sealed vials, were irradiated in duplicate by cathode rays produced at 3000 kv., for dosages of cathode rays varying from 0.17×10^6 to 5.28×10^6 roentgens-equivalent-physical (rep) (1). After irradiation, one set of samples, including a control, was assayed for niacin content chemically, and a duplicate set of samples was sent to Los Alamos for radioactivity determinations. The chemical assay results are presented in Table I. The results of the radioactivity assays are presented in tabular form in Table II and graphically in Fig. 1. The radioactivity assays were made of both the gaseous and the liquid phases in the ampul, as described above.

Discussion of Results

Niacin is decarboxylated by cathode rays, even at relatively low dosages (Table II). Splitting of the pyridine ring, as shown by chemical assays, does not begin to occur until the niacin has been bombarded by 0.66×10^6 rep of cathode rays, whereas 28 per cent of the niacin has been decarboxylated by 0.17×10^6 rep. The greater percentage of the decarboxylation occurs with the lower dosages of radiation. Hence the curve for decarboxylation referred to dosage flattens out in the region of dosages higher than 1.98×10^6 rep.

The spectrophotometric data indicate also that the ring is relatively stable, but a complete parallel between the chemical assay and the spectrophotometric assay is not to be expected, for one specific compound (niacin) is measured by the chemical method, whereas the spectrophotometric absorption curves are merely a resultant of all the breakdown products of niacin by the irradiation.

SUMMARY

1. Niacin labeled with C^{14} in the carboxyl group was subjected to irradiation by high voltage cathode rays, and the effect of these radiations on the niacin was measured by both chemical and radioactivity assays.

2. When niacin in a concentration of 100 γ per ml. was irradiated, decarboxylation to the extent of 28 per cent occurred with as little as 0.17×10^6 rep of cathode rays, whereas splitting of the pyridine ring did not begin to occur until 0.66×10^6 rep of cathode rays had been applied.

3. The rate of decarboxylation was increased with increments in dosage at lower levels but was less marked at dosages above 1.98×10^6 rep.

4. Splitting of the pyridine ring appeared to require irradiation of greater magnitude than decarboxylation.

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THE RESOLUTION OF SEVERAL RACEMIC AMINO ACIDS

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L- and D-alanine have been prepared in quantity and with a high degree of optical purity by a simple and convenient method based upon the following (1): (*a*) rat and hog kidney possess an enzyme which acts rapidly and asymmetrically upon *N*-acylated DL-alanine, hydrolyzing the acyl radical completely from the L form, and leaving the *N*-acylated D form intact; (*b*) from such a digest, the free L-amino acid can be separated by addition of alcohol, leaving the soluble acyl-D-alanine in the mother liquor; and (*c*) hydrolysis of the separated acyl-D-alanine by hot mineral acid, followed by neutralization, yields the free D-amino acid.

The asymmetric action of the kidney preparations with *N*-acylated derivatives of several other amino acids suggested that this method might be extended to the resolution of amino acids other than alanine (1). The present communication describes such an extension to the resolution of the isomers of racemic methionine, valine, threonine, isoleucine, serine, leucine, and aspartic and glutamic acids. The resolution of the basic and aromatic-substituted amino acids, as well as that of proline, presents special problems and will be described later.

EXPERIMENTAL

Since the method of resolution is substantially the same for all of the racemic amino acids so far studied, only a general procedure need be given. No particular difficulties have been encountered in the procedure as outlined, each step proceeding quite smoothly.

N-Acylation of Amino Acids—The rate at which a given kidney preparation will attack a number of *N*-acylated amino acids depends upon at least two factors, namely (*a*) the nature of the acyl radical, and (*b*) the nature of the amino acid. Thus, the rates of hydrolysis in terms of μM of substrate cleaved per hour per mg. of N (1) for the formyl, acetyl, chloroacetyl, propionyl, and benzoyl derivatives of alanine are, respectively, 22, 203, 800, 220, and 2.[1] The chloroacetyl appears to be the most susceptible of the derivatives studied (*cf.* also (1)) and is hydrolyzed roughly 3 to 4 times more rapidly than the corresponding acetyl derivative. Where chloroacetylation is convenient, it is recommended that this procedure be employed, and the chloroacetyl-DL-amino acid used as the starting material for enzymatic resolution. On the other hand, acetylation is cheaper and sometimes more convenient (as in the Knoop and Blanco (2) procedure), and when the rates of hydrolysis of the acetyl-L-amino acids are very high, as in the case of alanine, methionine, and leucine, it may be preferable to employ the acetyl-DL-amino acid as starting material for the resolution.

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[1] Personal communication from Dr. P. J. Fodor.

We have employed acetyl-DL-alanine (1) and acetyl-DL-methionine (1) prepared by the Knoop and Blanco procedure (2) which permits the easy accumulation of large stocks of these starting materials. All of the other amino acids studied were employed as the chloroacetylated derivatives. It is necessary to recrystallize these derivatives carefully from the appropriate organic solvents (generally acetone or ethyl acetate) in order to avoid introduction into the reaction mixture of any unchanged amino acid.

The preparation of chloroacetyl-DL-serine (3), chloroacetyl-DL-threonine (1), chloroacetyl-DL-glutamic acid (4), and chloroacetyl-DL-leucine (5) has been described. DL-Valine, DL-isoleucine, and DL-aspartic acid were chloroacetylated in the usual manner with chloroacetyl chloride in alkaline solution, yielding products which after crystallization from acetone possessed melting points, respectively, of 132°, 117°, and 149°, and N values of 7.2 (theory 7.2), 6.7 (theory 6.7), and 6.6 (theory 6.7).

With the same acyl radical, and under identical experimental conditions, the rates of hydrolysis of different amino acids vary greatly (1). The rates of hydrolysis of acetyl-DL-leucine (1), acetyl-DL-isoleucine (1), and of acetyl-DL-norleucine (m.p. 112°, N found 7.9, calculated 8.1), in terms of μM cleaved per hour per mg. of N are, for crude hog kidney aqueous extract, 250, 16, and 630 respectively, and, for a purified hog kidney enzyme preparation (see below), 2270, 62, and 5500. For various acetylated amino acids studied so far, the order of susceptibility to enzymatic hydrolysis by hog kidney extract is, in descending order, methionine, norleucine = alanine, glutamic acid, valine, arginine, isoleucine, and histidine (1). For the chloroacetylated compounds studied, the values for the rate of hydrolysis progressively decrease in the following order: leucine = alanine, serine, glutamic acid, aspartic acid, valine, threonine, isoleucine, and phenylalanine. Although we have invariably employed a purified enzyme preparation from hog kidney for all resolutions, it is probable that a crude, dialyzed aqueous kidney extract could be conveniently used for the resolution of the more susceptible compounds. For the less susceptible compounds, a purified enzyme preparation is essential.

Preparation of Partially Purified Enzyme—The detailed description of the preparation of an active concentrate of the hog kidney activity has been given (1). The enzyme preparation is essentially recovered as a sediment which is soluble in 15 per cent alcohol at pH 5.7 at −7° and which is insoluble at pH 5.1 in the presence of 30 per cent alcohol at −15°. A 6- to 10-fold increase in activity toward acylated DL-alanine over the crude tissue extract is usually achieved. In the course of these studies it has been assumed that this concentration in activity was approximately effective against all acylated amino acids (except proline).

Isolation of L-Amino Acids—The procedure follows essentially that described in the case of alanine (1) with a few modifications. 1 mole of the acylated racemic amino acid is dissolved or suspended in 100 cc. of distilled water, chilled, and treated with 6 N lithium hydroxide to pH 7.2 to 7.6. The solution is warmed to 37° and treated with enough purified enzyme solution to insure complete hydrolysis of the L form of the racemate in a few hours. The amount of enzyme needed is calculated from the observed rates of hydrolysis of the substrates by crude hog kidney extract corrected for the degree of concentration of the enzyme.[2] The pH of the digestion mixture is adjusted to 7.6, and samples of the digest taken in the course of the digestion period for determination of carboxyl nitrogen by the ninhydrin-CO_2 procedure. When about 80 per cent of the susceptible form has been hydrolyzed, fresh enzyme is usually added and the digestion carried for several hours beyond the point at which complete hydrolysis has been achieved. This is simply to insure complete hydrolysis, for unless this is accomplished the D-amino acid isolated at the end will contain some of the L form. Our customary practice is to set up such a digestion in the evening and allow it to run overnight. By early morning, the digestion is usually over. About 50 to 150 gm. of the acylated amino acids are used at a time, although this amount could easily be increased.

At the end of the digestion period, the mixture is acidified to pH 5.0 with glacial acetic acid when the monoaminomonocarboxylic acids are employed. For the resolution of glutamic and aspartic acids the mixture is acidified to pH 3.2 with 85 per cent lactic acid. The acidified mixture is then shaken for 1 hour with norit, filtered, and evaporated *in vacuo* at 40° to a very low bulk. The residue is transferred with the aid of a little water to a flask and treated with an excess of absolute alcohol. After a few hours of standing at 5°, the L-amino acid is filtered by suction, washed several times with hot alcohol, and recrystallized from hot water and alcohol. The mother liquor and washings containing the acyl-D-amino acid are combined and set aside for the preparation of the D-amino acid.

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[2] Rate values calculated as before (1) in terms of μM of substrate hydrolyzed per hour per mg. of N in the crude hog kidney extract are for chloroacetyl-DL-valine 185, chloroacetyl-DL-leucine 800, chloroacetyl-DL-isoleucine 46, chloroacetyl-DL-glutamic acid 385, and chloroacetyl-DL-aspartic acid 96.

Occasionally, some protein escapes the initial norit treatment and appears in the L-amino acid fraction. A second treatment with norit when recrystallizing the L-amino acid serves to remove all trace of protein. The L-amino acid so obtained is washed with alcohol and ether and dried *in vacuo* over P_2O_5 at 78° and 1 mm. of Hg pressure. The yields vary from 40 to 75 per cent, based upon the weight of acylated racemic amino acid taken.

Isolation of D-*Amino Acids*—The mother liquor with combined washings from the separation of the L-amino acids are brought to pH 1.0 to 2.0 by addition of concentrated hydrochloric acid and are evaporated *in vacuo* at 25° to dryness. The residue is taken up in the minimum amount of cold water and the aqueous mixture extracted 5 times with ethyl acetate. The acyl-D-amino acids pass into the ethyl acetate layer leaving salts and residual L-amino acid in the aqueous solution. The combined extracts are dried over anhydrous sodium sulfate and evaporated *in vacuo* to dryness. The residue is washed several times with petroleum ether to remove any free acetic or chloroacetic acid. In order to remove any trace of contamination by L-amino acids, the residue is again dissolved in acetone, filtered, and evaporated to dryness. No attempt was made to isolate and characterize the acyl-D-amino acids which frequently crystallize at this stage. Instead, the residue is taken up in 10 times its weight of 2 N hydrochloric acid and refluxed for 2 hours. This period was found by Van Slyke ninhydrin analysis to be sufficient for complete hydrolysis of the acyl-D-amino acids. The solution is treated with a small amount of norit, filtered, and evaporated *in vacuo* to dryness. Excess hydrochloric acid is removed by two subsequent evaporations with water. The residue is finally taken up in the minimum amount of cold water, chilled further, and treated dropwise and with stirring with 4 N lithium hydroxide. In the case of the monoaminomonocarboxylic acids, the pH is adjusted to 5.0. In the case of glutamic and aspartic acids the pH is adjusted to 3.2. An excess of absolute alcohol is added to precipitate the free D-amino acid. After standing several hours at 5°, the amino acid is filtered and purified by recrystallization in the same fashion as the L isomer. The yields vary from 30 to 60 per cent, based upon the amount of acylated racemic amino acid taken.

Resolution Data on Amino Acids—The optical data on the amino acids prepared by the resolution procedure described are given in Table I. The results compare favorably with those of other investigators obtained by different methods. The preparation of optically pure isomers of threonine and of isoleucine suggests that either the respective DL-amino acids possessed inappreciable amounts of the allo forms, or else that these forms were removed somewhere in the course of the reactions.[3]

The advantages of our procedure are simplicity and rapidity of operation and the possibility of working up nearly unlimited quantities of material. From 8 to 10 kilos of fresh hog kidneys it is possible in 5 to 8 days to prepare an enzyme concentrate which will hydrolyze in a few hours at 37° about 100 moles of acylated racemic methionine. By suitably enlarging the scope and operational procedures of the method, there

TABLE I

Specific Rotations at 25° of Optical Isomers of Racemic Amino Acids Resolved by Asymmetric Enzymatic Hydrolysis of Their N-Acylated Derivatives

The numbers in parentheses refer to the bibliography.

Amino acid	Present data					Data in literature	
	L form		D form		N calculated	L form, $[\alpha]_D$	D form, $[\alpha]_D$
	$[\alpha]_D$	N observed	$[\alpha]_D$	N observed			
Alanine (1)	+14.4[a]	15.7	−14.4[b]	15.7	15.7	+14.7 (6), +14.6 (7)	−14.5 (7)
Methionine	+21.6[c]	9.4	−21.5[d]	9.4	9.4	+20.7 (8)	−21.2 (9), −21.5 (8)
Valine	+27.4[e]	11.9	−27.1[f]	11.9	12.0	+28.8 (10)	−29.0 (10), −27.6 (11)
Threonine	−28.5[g]	11.6	+28.5[g]	11.6	11.8	−28.3 (12)	+28.4 (12)
Isoleucine	+40.7[h]	10.6	−40.4[h]	10.6	10.7	+40.6 (13)	−40.9 (13)
Serine	+14.9[i]	13.3	−15.0[i]	13.3	13.3	+14.5 (14)	−14.3 (14)
Leucine	+15.9[j]	10.7	−15.6[k]	10.7	10.7	+15.8 (15)	−15.6 (15)
Glutamic acid	+32.0[l]	9.4	−31.9[m]	9.3	9.5	+32.0 (16), +32.2 (17)	−30.1 (16), −32.0 (18)
Aspartic acid	+25.2[n]	10.4	−25.3[n]	10.5	10.5	+25.1 (17)	−25.5 (16)

[a] 1.615 gm. in 25 cc. of 1 N HCl.
[b] 1.592 gm. in 25 cc. of 1 N HCl.
[c] 0.221 gm. in 25 cc. of 0.2 N HCl.
[d] 0.223 gm. in 25 cc. of 0.2 N HCl.
[e] 0.249 gm. in 25 cc. of 6 N HCl.
[f] 0.208 gm. in 25 cc. of 6 N HCl.
[g] 0.500 gm. in 25 cc. of water.
[h] 0.620 gm. in 25 cc. of 6 N HCl.
[i] 1.000 gm. in 25 cc. of 1 N HCl.
[j] 0.954 gm. in 25 cc. of 6 N HCl.
[k] 0.842 gm. in 25 cc. of 6 N HCl.
[l] 0.520 gm. in 25 cc. of 6 N HCl.
[m] 0.515 gm. in 25 cc. of 6 N HCl.
[n] 0.500 gm. in 25 cc. of 6 N HCl.

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[3] Racemic alanine and isoleucine were Merck products. Threonine was obtained from the Interchemical Corporation, valine from the Winthrop-Stearns, Inc., aspartic acid from Eastman Kodak, and methionine, leucine, serine, and glutamic acid from the Nutritional Biochemicals Corporation.

is no reason why the yields of amino acid isomers may not be measured in terms of kilos and at relatively low cost. This is particularly true if only the L isomer is wanted.

The authors are indebted to Mr. Robert Koegel for the nitrogen analyses.

SUMMARY

The method of resolving *N*-acylated racemic amino acids by means of asymmetric enzymatic hydrolysis of the L-acylated isomer, previously described for the case of alanine, has been extended to include the resolution of racemic methionine, valine, threonine, isoleucine, serine, leucine, and aspartic and glutamic acids.

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HEMATOLOGIC EFFECT IN RATS OF PTERINS STRUCTURALLY RELATED TO PTEROYLGLUTAMIC ACID*

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The realization that there are synthetic and naturally occurring substances which inhibit the metabolic action of certain compounds has led to the more or less systematic quest for these inhibitors as they relate to a variety of metabolites. The current status of this work has been reviewed by Woolley (1).

Since pteroylglutamic acid (PGA) is essential for hematopoiesis in many animal species, the biologically effective inhibitors of this vitamin would be expected to cause a depression in cellular elements of the peripheral blood (2). Synthetic products structurally related to PGA have now been described which are capable of influencing the hematopoietic process in a number of different animals, including man (3–12). Of the compounds studied thus far 4-amino-PGA (pteroylglutamic acid having the hydroxyl group at position 4 replaced by an amino group) has proved to be the most potent inhibitor. For this reason we have thought it of interest to report some experiments which compare the hematologic effect in rats of several pterins structurally related to pteroylglutamic acid with various substituents at the 4 position.

Since the general basis for selection of these particular compounds was their bacterial inhibition index, a comparison will also be made of the inhibition indices of these pterins on bacteria requiring a nutrient source of PGA.

It will be seen that in this study pterins without the *p*-aminobenzoylglutamic acid group but with a 4-amino substituent are relatively less inhibitory towards *Lactobacillus casei* than *Streptococcus faecalis*. In rats, under conditions of the experiment, these pterins appear to depress leucocyte formation more than the formation of red blood cells, whereas pterins with a *p*-aminobenzoylglutamic acid group as well as a substituted group at position 4 cause both an anemia and a leucopenia.

An incidental result of the study has been to show that in several instances pterins which at a high dietary level cause leucopenia at a lower dietary level actually serve, either directly or indirectly, to maintain leucocytes within a normal range.

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EXPERIMENTAL

Compounds Used—The synthetic pterins which were used in these studies, 2-amino-4-hydroxy-6,7-diphenylpteridine, 2,4-diamino-6,7-diphenylpteridine, and 2,4-diamino-6,7-dimethylpteridine, were prepared essentially as described by Mallette *et al.* (13) and Cain *et al.* (14). The 4-amino-PGA, (*N*[4-{([2,4-diaminopteridyl-6]methyl)amino}benzoyl]glutamic acid), was prepared by condensing the reaction product of *p*-aminobenzoylglutamic acid and α-bromoacrolein with 2,4,5,6-tetraminopyrimidine. The crude reaction product was purified by a procedure similar to that used for pteroylglutamic acid (15). An analytically pure sample of the magnesium salt $C_{19}H_{18}O_5N_8Mg \cdot 3H_2O$ gave an inhibition index of 2 for *S. faecalis* at a concentration of pteroylglutamic acid of 0.005 γ per 10 cc. This calculates to an inhibition index of 1.7 for the free acid. Highly purified samples of the free acid gave inhibition indices ranging from 0.8 to 2. In this work a sample of 4-amino-PGA of inhibition index of 2 was used. The 4-desoxypteroylglutamic acid was prepared by condensing the reaction product of *p*-aminobenzoylglutamic acid and α-bromoacrolein with 2,4,5-triaminopyrimidine. The crude reaction product was used without purification.

Experiments with Bacteria—The effect of the pterins mentioned on the growth of *S. faecalis* and *L. casei*, both of which require a nutrient source of PGA, was determined. The medium used for the growth of *S. faecalis* was that of Teply and Elvehjem (16). *L. casei* was grown on the medium of Mitchell and Snell (17). For each compound investigated, the inhibition index was determined; that is, the ratio of inhibitor concentration to PGA concentration at which half inhibition of growth of the organism occurs. The general procedure was to set up a series of tubes with a constant amount of PGA (0.5 mμgm. per tube for *L. casei* and 5 mμgm. per tube for *S. faecalis*) and varying amounts of inhibitor. After the incubation period, growth was measured turbidimetrically and the point of half inhibition of growth was obtained.

Experiments with Rats—The pterins were administered to weanling rats to determine whether they produced the hematologic pattern, anemia with accompanying leucopenia, that is characteristic of PGA deficiency.

In order to facilitate comparison of the hematologic effects of these compounds the following assay procedure was adopted: Weanling rats were placed on a basal diet of the following percentage composition: purified casein 18, cerelose 66, Jones and Foster salt mixture (18) 4, cottonseed oil 6, corn oil 2, "sulfasuxidine" 2, thiamine 0.014, riboflavin 0.014, pyridoxine

0.014, pantothenic acid 0.18, nicotinic acid 0.18, inositol 0.18, and choline 1.08. The rats were treated with 5500 units of vitamin A, 500 units of vitamin D, and 7 mg. of mixed tocopherol orally once a week. This diet was supplemented with varying amounts of the pterin under investigation. The test period was standardized at 14 days. At the end of that time hemoglobin concentrations were determined and leucocyte counts made (19).

Results

In Table I, inhibition indices for the various pterins are reported with *S. faecalis* and *L. casei* as the test organisms. These indices are on the basis of a weight ratio, since the pterins were also to be compared from the standpoint of weight in animal feeding experiments. It can be calculated that on the basis of a molecular ratio the indices for the pterins not containing the *p*-aminobenzoylglutamic acid substituent would be somewhat higher.

TABLE I

Effect of 4-Substituted Pterins on Bacterial Growth

Substituted pterin	Inhibition index*	
	S. faecalis†	*L. casei*‡
2,4-Diamino-6,7-diphenylpteridine	10	200,000
2,4-Diamino-6,7-methylpteridine	5,000	50,000
2-Amino-4-hydroxy-6,7-diphenylpteridine	40,000	200,000
4-Aminopteroylglutamic acid	2	8
4-Desoxypteroylglutamic acid (crude)	30	50

* Weight ratio of substituted pterin to PGA at which half inhibition of growth of the organism occurs.

† 5 mμgm. of PGA per tube.

‡ 0.5 mμgm. of PGA per tube.

It was found that the pterins not containing the *p*-aminobenzoylglutamic acid group were much more effective in inhibiting the growth of *S. faecalis* than that of *L. casei*. The most potent of these compounds, as an inhibitor for the former organism, was 2,4-diamino-6,7-diphenylpteridine with an inhibition index of 10.

Those pterins containing the *p*-aminobenzoylglutamic acid group (4-amino-PGA and 4-desoxy-PGA) have approximately the same inhibition index for *S. faecalis* as for *L. casei*. The efficacy of 4-desoxy-PGA as a bacterial growth inhibitor cannot be properly evaluated, since it was not used in the pure form.

In rat studies, the level at which 2,4-diamino-6,7-diphenylpteridine exerted a hematologic effect was first determined. It will be seen from Table II that a dietary concentration of 50 mg. per cent of this compound produced a leucopenia with agranulocytosis but had no effect on hemoglobin concentration. The leucopenia could be prevented by the simultaneous inclusion in the diet of equivalent amounts of PGA. At this same dietary concentration of 50 mg. per cent, the two pterins, 2-amino-4-hydroxy-6,7-diphenylpteridine and 2,4-diamino-6,7-dimethylpteridine, had no effect on the hematologic pattern. Thus substitution of a hydroxy for an amino group in the 4 position or substitution of methyl groups for phenyl groups in the 6 and 7 positions reduces the inhibitory effect of the pterin. When the dietary concentration of 2,4-diamino-6,7-dimethylpteridine was increased ten times, it did have an effect on the blood picture similar to that of 2,4-diamino-6,7-diphenylpteridine in that only the leucocyte level was lowered, while the hemoglobin concentration remained the same. It was further found that, with animals fed these pterins capable of depressing leucocyte levels, death would ensue, while hemoglobin concentrations were still in the normal range.

TABLE II

Hematologic Effect on Rat of Some 4-Substituted Pterins

Supplement in ration	Concentration	Total leucocytes*	Granulocytes*	Hemoglobin*
	mg. per cent	*per c.mm.*	*per c.mm.*	*gm. per 100 cc.*
None		12,400	2200	13.7
2,4-Diamino-6,7-diphenylpteridine	50	4,200	150	14.1
50 mg. % PGA with 2,4-diamino-6,7-diphenylpteridine	50	12,800	2000	13.8
2,4-Diamino-6,7-dimethylpteridine	50	12,700	3350	13.0
"	500	5,300	650	13.2
2-Amino-4-hydroxy-6,7-diphenylpteridine	50	9,700	1850	13.8
4-Aminopteroylglutamic acid	0.3	6,200	250	9.1
0.3 mg. % PGA with 4-aminopteroylglutamic acid	0.3	11,150	2100	14.0
4-Desoxypteroylglutamic acid (crude)	500	7,250	750	7.5

* The values represent the average obtained with six rats; experimental period, 2 weeks.

When 4-amino-PGA was investigated, it was found (Table II) that, concomitant with the development of leucopenia with agranulocytosis, the hemoglobin concentration was also lowered. The anemia which developed was found generally to be normocytic and normochromic. These hematologic changes occurred when 4-amino-PGA was present in the diet in the amount of 0.3 mg. per cent. When the dietary concentration was doubled, the rats would not survive for the test period of 14 days. The hematologic changes, as shown in Table II, were completely prevented by the dietary addition of PGA in amounts equivalent to 4-amino-PGA. Data obtained with crude 4-desoxy-PGA show that this compound acts similarly to 4-amino-PGA to produce both agranulocytosis and a reduction in hemoglobin concentration. However, a much greater amount of 4-desoxy-PGA, 500 mg. per cent in the diet, is necessary to cause this change in the blood picture.

In Table III data are presented on some experiments of 5 weeks duration. Rats fed the basal diet containing "sulfasuxidine" develop agranulocytosis during this period (20). When PGA inhibitors are added, however, at concentrations too low to cause any leucocyte reduction at a 2 weeks interval, this agranulocytosis is prevented. With both 4-desoxy-PGA and 2,4-diamino-6,7-dimethylpteridine, the granulocyte as well as the total leucocyte level is within or higher than the normal range. It appears, therefore, that at least some PGA inhibitors at low concentrations are capable of stimulating leucocyte production.

TABLE III

Effect of Low Concentrations of Some 4-Substituted Pterins on Leucocytes of Rat

Supplement in ration	Amount	Total leucocytes*	Granulocytes*
	mg. per cent	*per c.mm.*	*per c.mm.*
None		9,050	550
4-Desoxy-PGA	100	12,000	2900
2,4-Diamino-6,7-dimethylpteridine	50	23,500	6850

* The values represent the averages obtained on six rats; experimental period, 5 weeks.

DISCUSSION

Daniel *et al.* (6) have made an extensive study concerning the inhibition of bacterial growth by a series of pterins which did not have a *p*-aminobenzoylglutamic acid substituent. Our results with these same compounds are very similar to theirs. When they extended their study to chicks (21), they observed that several of the pterins caused a pronounced lowering of hemoglobin concentration. The effect on leucocytes was not mentioned. In our experiments with the rats, which were fed a basal diet, free from PGA and supplemented with "sulfasuxidine," we observed that with compounds of this type changes in the peripheral blood were confined to leucocyte formation. These differences between our experimental results and those of Daniel and coworkers might well be due to a species difference, since avian blood morphology varies greatly from that of the rat.

The preferential effect on rat leucocytes of 4-amino-substituted pterins without the *p*-aminobenzoylglutamic acid group has been demonstrated for two different compounds. The possibility is suggested that breakdown products of PGA comprising only a portion of its molecule might function to some degree in the maturation of the leucocyte, but not in red blood cell formation. This suggestion gains credence from a consideration of the data on bacterial growth where it has been shown that the 4-amino-substituted pterins without the *p*-aminobenzoylglutamic acid residue are most effective as inhibitors for *Streptococcus faecalis*, which does not require the complete PGA molecule for growth (22).

It has been observed that certain antimetabolites, which in larger amounts inhibit bacterial growth, when present in smaller amounts actually stimulate bacterial growth (1). A somewhat analogous situation would appear to result with regard to rat leucocytes when low concentrations of certain of the PGA inhibitors are fed, since the leucocyte levels are maintained at a normal or elevated range when granulocytopenia has developed in control animals not fed PGA inhibitors. It may be that these PGA inhibitors act to increase leucocyte production indirectly through their stimulation or depression of the as yet unknown factors which regulate cell marrow release and cell destruction.

Some data have been given showing that PGA can prevent the hematologic effect of the 4-amino-substituted pterins studied here. This is, of course, additional evidence that these pterins exert their influence on blood cell formation by interfering with the function of PGA.

From their bacterial growth study, Daniel and coworkers (6) have shown the importance of the 4 position group on the pteridine ring in the PGA molecule. In the rat as well, it would appear that the 4 position group on the pteridine ring has a key function in enabling PGA to assume its proper metabolic rôle.

SUMMARY

Some 4-substituted pterins, structurally related to pteroylglutamic acid, have been compared as to their effects on the growth of bacteria requiring a nutrient source of PGA and on the production of blood cells in the rat fed a purified diet, free from PGA and supplemented with "sulfasuxidine."

Those pterins not containing the *p*-aminobenzoylglutamic acid group had a lower inhibition index for *Streptococcus faecalis* than for *Lactobacillus casei*. The most effective inhibitor of this type of compound, 2,4-diamino-

6,7-diphenylpteridine, when fed to rats, caused a leucopenia but no reduction in hemoglobin. The other compounds tested had no such effect when fed at the same level.

The compounds 4-amino-PGA and 4-desoxy-PGA inhibited growth of both *S. faecalis* and *L. casei*. These compounds when fed to rats caused anemia as well as leucopenia. 4-Amino-PGA was the most potent inhibitor both as to its effect on bacterial growth and on the hematologic pattern in rats.

The effects of the 4-substituted pterins on the blood picture of rats can be prevented by the dietary addition of PGA.

Some of the 4-substituted pterins, which cause leucopenia at high dietary levels, at a lower dietary level stimulate leucocyte production.

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BIOSYNTHESIS OF UREA

I. ENZYMATIC MECHANISM OF ARGININE SYNTHESIS FROM CITRULLINE*

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In urea synthesis, according to the Krebs-Henseleit ornithine cycle (1), the transfer of nitrogen to citrulline to form arginine (Step II) was thought to occur from NH_3. It was not until Cohen and Hayano (2) succeeded in demonstrating rapid arginine synthesis in liver homogenates from glutamic acid and citrulline that the transfer from an amino acid rather than from NH_3 was recognized as a main pathway in urea formation. Under their conditions, arginine was formed in the presence of oxygen, Mg^{++}, and catalytic amounts of adenosine triphosphate (ATP). This was the same reaction observed earlier by Borsook and Dubnoff (3) in kidney slices and considered at the time to be a transamination with simultaneous oxidative removal of 2 H atoms (transimination). An α-keto acid, supposedly the second product of the reaction, would of course escape detection in respiring preparations. Whereas in the kidney slice experiments of Borsook and Dubnoff, both aspartic and glutamic acid were equally effective as amino group donors, Cohen and Hayano found in liver homogenates that glutamic acid was about 4 times as effective as aspartic acid. They concluded therefore that glutamic acid was the specific donor in the Borsook-Dubnoff reaction, aspartic acid being utilized only to the extent that it could be converted to glutamic acid.

This investigation was undertaken to elucidate further some fundamental aspects of the enzymatic mechanism involved.

As briefly reported earlier, it has been possible to obtain arginine synthesis with a partially purified enzyme system prepared by alcohol fractionation of acetone powder extracts of mammalian liver (4). In the isolated system, aspartic acid and citrulline are converted to arginine and malic acid in the presence of Mg^{++}. ATP participates directly as a reactant and the reaction proceeds anaerobically without involving the transport of H atoms. In view of the evidence presented below, the transfer

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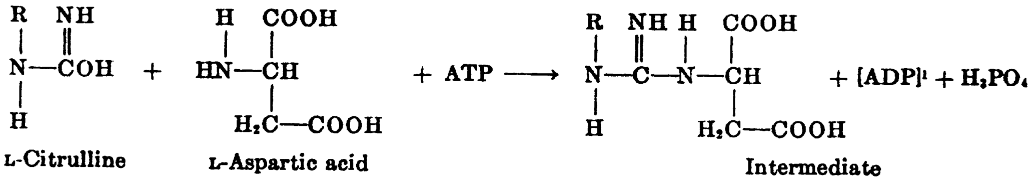
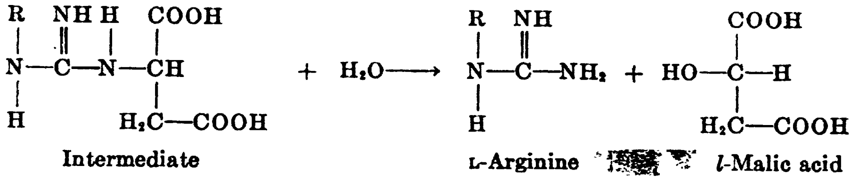
(1, *a*)



(1, *b*)



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[1] The ability of ADP to act as the ∼ph donor in this reaction is not yet known.

of nitrogen is formulated as an exchange between the OH group of the isourea form of citrulline and the amino group of aspartic acid through formation of an intermediary condensation product, as shown in Reaction 1.

Intermediary Condensation Product—That malic acid formation is actually associated with arginine synthesis is shown by the following evidence: Neither product of the reaction appears unless both citrulline and aspartic acid are present simultaneously; malic acid always appears in amounts equivalent to the arginine formed; malic acid cannot be formed from aspartic acid independently. It may be seen from Table I that with the complete system (citrulline, aspartic acid, ATP, generated from phosphoglyceric acid, and Mg^{++}) an equal amount of arginine and malic acid was formed in the 20 minute period, while in the absence of either aspartic acid or citrulline no reaction occurred. It is especially significant that, when citrulline was omitted (Table I, Line 2), malate was not formed from aspartic acid. The possibility that malic acid might be formed by a separate reaction was tested further. When an alcohol-fractionated and thoroughly dialyzed preparation was used as the enzyme source, such as that used to obtain the data shown in Table I, neither malic acid nor arginine was formed when aspartic acid was replaced by NH_3 or by the combination of NH_3 and oxalacetic acid. This may be taken not only as evidence that NH_3 is unreactive in this system, but also that malic acid cannot be formed by reduction from oxalacetate. Further proof is afforded by evidence that any pyridine nucleotides which might participate in hydrogen transport have been removed. The preparation employed contains some malic dehydrogenase and glutamic dehydrogenase. When oxalacetic acid and glutamic acid were present together, in the absence of citrulline, no malic acid was formed (Table I, Line 6). These dehydrogenases are known (5) to be capable of catalyzing the following dismutation: glutamate + oxalacetate $\rightleftharpoons$ α-ketoglutarate + NH_3 + malate. Under such conditions malate should have appeared if diphosphopyridine nucleotide (DPN) had been present in sufficient concentration.[2] It is therefore evident that under our experimental conditions the system is incapable of forming malic acid by reactions other than that involving interaction between citrulline and aspartic acid, even when oxalacetate and a source of H atoms are supplied.

The fact that arginine and malic acid are formed simultaneously from the interaction of the two substrates strongly suggests that the mechanism of nitrogen transfer involves a preliminary condensation of the amino group of aspartic acid with the ureido C of citrulline to form a C—NH—C linkage, followed by cleavage on the second side of the nitrogen, as shown in Reaction 1.

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[2] See the last section of Paper II concerning the DPN dependence of this reaction.

The question arises as to whether Reaction 1 is mediated by a single enzyme, with transient formation of the intermediate such as is postulated in transamination between keto and amino acids (6, 7), or whether it proceeds in a stepwise manner. Very recently two enzymes have been separated by repeated ammonium sulfate fractionation of acetone powder extracts of ox liver (8). One of them catalyzed Reaction 1, *a*, as shown by the disappearance of citrulline and the simultaneous appearance of inorganic phosphate. When the resulting deproteinized reaction mixture was incubated with the second enzyme, arginine and malic acid were formed simultaneously as shown in Reaction 1, *b*, by what appears to be a purely hydrolytic step. A detailed study of the two enzymes is now in progress and will be reported at a later date. The preliminary results indicate, however, that, under suitable conditions, accumulation of the postulated intermediate can be demonstrated, and that the transfer of the —NH_2 group proceeds by at least two enzymatic steps.

TABLE I

Synthesis of Arginine from Citrulline in Alcohol-Fractionated Extract of Ox Liver Acetone Powder

Each tube contained in addition 4 μM of ATP, 13 μM of $MgSO_4$, 0.4 ml. of phosphate buffer, pH 7.5, enzyme preparation containing 31 mg. of protein and 8 mg. of muscle extract in a final volume of 4 ml. Time 20 minutes; 38°.

Substrate added					Found	
L-Aspartate, 20 μM	L-Glutamate, 20 μM	Oxalacetate, 30 μM	L-Citrulline, 20 μM	3-Phosphoglycerate, 50 μM	Arginine	*l*-Malic acid
					μM	μM
+			+	+	14.4	14.5
+				+	0.0	0.0
+			+		0.9	1.0
	+	+	+	+	7.0	6.8
	+		+	+	0.0	0.0
	+	+		+	0.0	0.1
		+	+	+	0.0	0.2

Rôle of ATP—Unlike keto-amino transamination, high energy phosphate (~ph)[3] is utilized in Reaction 1 and the phosphate transfer occurs specifically from ATP. For reasons which are not at present understood, ATP causes inhibition in concentrations above 5 × 10^{-3} M, but at this level 3 to 4 μM of arginine are formed in a 20 minute period from 10 μM of ATP as the only source of ∼ph, under conditions otherwise similar to those employed for obtaining the data given in Table I.

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[3] This symbol as a designation of the energy-rich phosphate bond follows the usage introduced by Lipmann (9).

Competition with contaminating adenosinetriphosphatase (ATPase), which was appreciable even in alcohol-fractionated preparations, introduced uncertainties in the investigation of the stoichiometric participation of ATP. However, recent evidence obtained from a preliminary study of Reaction 1, *a*, in which ammonium sulfate-fractionated enzyme was employed, indicates that inorganic phosphate and an intermediate product are formed in equivalent amounts. Whether phosphorylation precedes condensation or occurs simultaneously with it remains to be investigated.

Nature of Condensation—Sufficient data are not available to permit the calculation of the energy change in each step of the ornithine cycle, but it is reasonable to assume, in view of the endergonic nature of urea synthesis from NH_3 and CO_2, that citrulline and arginine formation are each endergonic, and that, as far as arginine synthesis is concerned, it is in the condensation Reaction 1, *a*, that energy, derived from ATP, is actually utilized.

The condensation product is a substituted guanidine, whereas a Schiff base type of compound is formulated as an intermediate in keto-amino transamination. It seems more probable that aspartic acid will condense with the isourea rather than the urea form of citrulline, since *O*-methyl and *S*-methyl isourea readily form guanidines with amines, chemically, whereas urea does not. Although information regarding the phosphorylation step is still lacking, phosphorylation of isocitrulline would appear to be more likely than aspartic acid phosphorylation, for it provides a means of shifting the citrulline equilibrium toward the isourea form. Once the intermediate is formed, hydrolytic cleavage of the C—N bond of the aspartic acid moiety would presumably be favored by the formation of the highly resonating monosubstituted guanidine group of arginine as compared to the more restricted resonance of the *N*, *N'*-disubstituted guanidine group of the intermediate (10).

Properties of Enzyme System

Phosphoglyceric Acid As Generator of ∼ph—Owing to competition with ATPase, the rate of the over-all reaction is low to a misleading degree, when ATP is used directly. In order to approach maximum rates, and at the same time to avoid the complications of ATP inhibition, it was found more feasible to employ ATP as the catalyst and phosphoglyceric acid as the source of ∼ph. In making use of phosphoglyceric acid as a source of ∼ph, advantage has been taken of the presence of phosphoglyceromutase

(11), enolase (12), and phosphoenol transphosphorylase in acetone powder extracts of liver. These enzymes are likely to be limiting in the crude extract and to become increasingly more so upon fractionation. They were therefore supplied in excess by addition of an ammonium sulfate fraction prepared from rabbit muscle extract. This muscle fraction increased the rate of arginine synthesis, without itself possessing arginine-synthesizing activity.

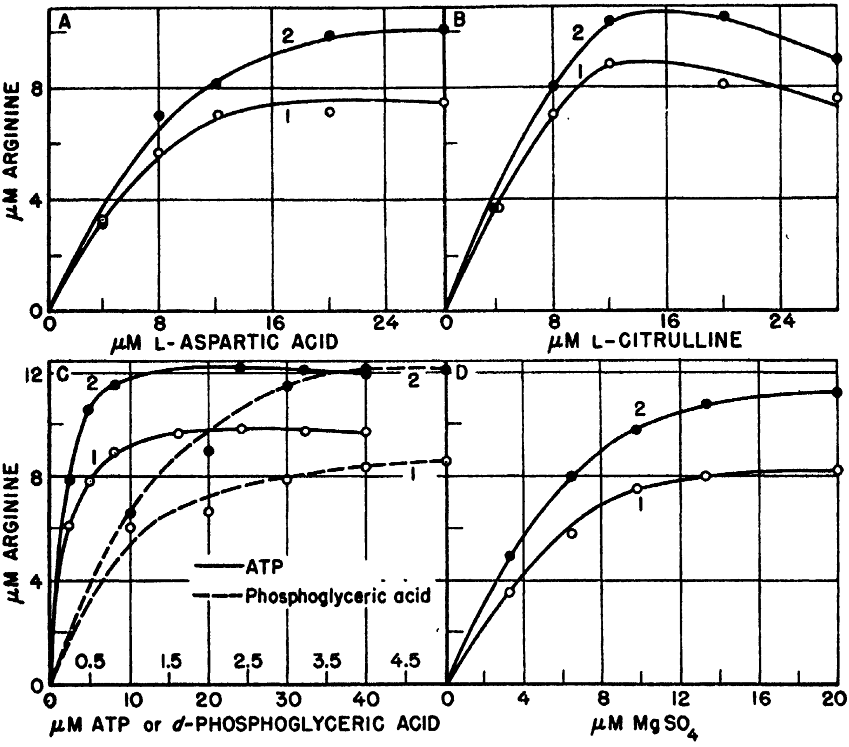

Fig. 1. Dependence of the rate of arginine synthesis on the concentration of L-aspartate, L-citrulline, magnesium sulfate, ATP (scale 0 to 5 µM), and 3-phosphoglycerate. In *A*, *B*, *C*, and *D*, Curve 1 represents acetone powder extract, 38 mg. of protein per tube; Curve 2, as in Curve 1, supplemented with muscle extract. The conditions are otherwise as in Table I.

The phosphoglyceric acid dependence curves (Fig. 1, *C*) show that a large excess was required in order to achieve maximum rates and that the addition of muscle extract caused a 35 per cent stimulation. The ATP curves in Fig. 1, *C* show that under these conditions ATP functioned catalytically; maximum rates were reached with 1.6 µM.

Optical Specificity—The enzyme system reacts only with the natural isomers of citrulline and aspartic acid. Optical specificity was investigated on a rate basis, in each case the activity of the DL isomer being compared with that of the L isomer at two concentrations. As shown in Table II, the rate of arginine synthesis is the same with 20 μM of DL-aspartic acid as with 10 μM of the L form and similarly with citrulline.

Identification of Products of Reaction—Although little doubt now remains that urea is formed exclusively from arginine, alternative pathways (see for example Bach (13)) have occasionally been proposed. Arginase was present in excess in all the enzyme preparations employed and arginine was therefore estimated as urea by the colorimetric method of Archibald (14). In order to establish with certainty that arginine was the primary product, ornithine was isolated from a large scale enzymatic run and identified as dibenzoyl-L-ornithine (see "Experimental"). *l*-Malic acid was identified enzymatically by means of a highly purified preparation of the "malic" enzyme of Ochoa, Mehler, and Kornberg (15), which acts only on the *l* form, and by isolation as cinchonine *l*-malate.

TABLE II

Optical Specificity of Aspartic Acid and Citrulline in Arginine Synthesis

The conditions are as in Table I, but without muscle extract.

Amino acid varied	Added	Arginine found		
		20 min.	40 min.	60 min.
	μM	μM	μM	μM
L-Citrulline	20	11.3	15.4	15.0
"	10	7.6	7.5	7.7
DL-Citrulline	20	7.5	7.5	7.5
L-Aspartate	20	12.0	15.4	15.8
"	10	7.6	8.4	8.0
DL-Aspartate	20	7.6	8.2	8.0

Substrate Specificity—Thus far no amino acid other than aspartic acid has been found to react with citrulline. The amino acids tried were DL-serine, DL-lysine, DL-isoleucine, L-leucine, L-proline, DL-alanine, DL-aminoadipic acid, DL-aminopimelic acid, L-tyrosine, L-histidine, DL-tryptophan, DL-ornithine, DL-valine, L-cysteine, glycine, DL-methionine, DL-threonine, DL-phenylalanine, and L-glutamic acid. They were all tested both with and without oxalacetic acid, at the concentration known to be optimum for aspartic acid, with a 40 minute incubation period and twice the amount of acetone powder extract ordinarily used.

Aspartic acid may be replaced by a combination of glutamic acid and oxalacetic acid, but not by glutamic acid alone (Table I, Lines 4 and 5).

The activity of this combination can be readily explained by the formation of aspartic acid by transamination. This has been corroborated by the assay of various preparations for glutamic-aspartic transaminase activity. Although the rate of aspartic acid appearance under the experimental conditions employed for arginine synthesis cannot be calculated from the assay data, the relationship can be shown by comparing, in several preparations, the rate at which arginine is formed by the combination of glutamic and oxalacetic acid, or aspartic acid, with the transaminase concentration of the preparation. For example, in 20 minutes, 0.5 ml. of a crude extract of ox liver acetone powder, containing 30 units of transaminase (see "Experimental"), catalyzed the formation of 8.1 μM of arginine with aspartic acid, and 7.2 μM with glutamic and oxalacetic acids, while 0.4 ml. of the alcohol-fractionated preparation used to obtain the data in Table I, containing 12 units of transaminase, catalyzed the formation of 14.4 μM of arginine with aspartic acid, and 7.0 μM with glutamic and oxalacetic acids, in the same period of time. In other words, the difference between the two rates is small when transaminase is present in excess, but very large when the transaminase concentration is limiting. Acetone powder extracts of rat liver were found to behave in an entirely similar way toward aspartic acid, toward glutamic acid, and toward the combination of glutamic and oxalacetic acids. Presumably the same may be said of mammalian liver in general.

If glutamic-alanine transaminase were also present in acetone powder extracts, the combination of alanine, oxalacetic acid, and a catalytic amount of α-ketoglutaric acid would be expected to form aspartic acid, as suggested by Green, Leloir, and Nocito (16) and demonstrated by O'Kane and Gunsalus (17). This combination, tested in acetone powder extracts, did not give rise to aspartic acid (as measured by arginine appearance) for the reason that glutamic-alanine transaminase, being low in liver tissue to start with, probably fails to survive acetone treatment.

The formation of arginine from α-aminoadipic acid and citrulline in kidney slices, reported by Dubnoff and Borsook (18), might be interpreted, in view of Braunstein's observation (19) that α-aminoadipic acid can transaminate with pyruvic acid, by the presence in slices of enzymes capable of transferring the amino group of α-aminoadipic acid to oxalacetate by transamination reactions. Acetone powder extracts were tested with α-aminoadipic acid and various combinations of oxalacetic acid, pyruvic acid, and α-ketoglutaric acid in the hope of coupling the two transaminating systems. The results were negative, as with alanine.

Substrate Dependence and Other Properties—The enzyme system, which is quite soluble, can be readily extracted from acetone powders of mammalian liver (rat, pig, ox) with water or dilute phosphate buffer, and is fairly stable to low temperature fractionation with ethyl alcohol or ammonium sulfate. The pH optimum of the over-all reaction lies between pH 7.4 and 7.5 in agreement with that found by Cohen and Hayano (20) for liver homogenates.

The requirement for Mg^{++} is rather high, as shown in Fig. 1, *D*, half saturation occurs with 3.8 μM of $MgSO_4$ with and without muscle extract, corresponding to 0.95×10^{-3} M. In addition to the known dependence upon Mg^{++} of ~ph transfer from phosphopyruvic to adenosine diphosphate, Mg^{++} is specifically required in the condensation Reaction 1, *a*. When ATP was the only ~ph donor, the reaction did not proceed in the absence of Mg^{++} and, in the presence of the latter, 0.01 M fluoride caused approximately 50 per cent inhibition. Studied separately, the condensing enzyme showed, in common with a number of other phosphate-transferring enzymes, the same specific requirement for Mg^{++} and inhibition by fluoride as the over-all system. These observations indicate in part the basis of the fluoride inhibition found in homogenates by Cohen and Hayano (2).

Citrulline and aspartic acid concentration dependence curves are shown in Fig. 1, *B* and 1, *A*. Half saturation of the enzyme system was reached in both cases with 4.8 μM (1.2×10^{-3} M) with and without added muscle extract.

Estimation of Enzyme Activity—In order to estimate the specific activity (units per mg. of protein) of various fractions, as purification proceeds, a unit has been chosen as the amount of enzyme which will catalyze the formation of 1 μM of arginine per hour under standardized test conditions, these being a 20 minute incubation period during which time no more than 10 μM of arginine have been formed from the 20 μM of citrulline and aspartic acid added. Under these conditions the rate of arginine formation is linear from 10 to 25 minutes, as shown in Fig. 2, *A*. An induction period may be noted in the first 10 minutes, undoubtedly due to the time lag necessary to reach an optimum concentration of intermediate, as might be expected in a stepwise reaction. It was not abolished by the addition of muscle extract and is therefore not likely to be associated with a limit in ATP formation. Above 25 minutes, the rate falls off, owing to declining substrate concentrations.

Regardless of the specific activity of the preparation, enzyme concentration dependence studies reveal a disproportionate increase in activity with increasing enzyme concentration. Fig. 2, *B* shows the rates obtained with three different preparations. Since muscle extract was added in excess, and separate experiments indicated that arginase was not a limiting factor, the exponential effect may be ascribed to the presence of the two enzyme components of Reaction 1, mentioned above.

The choice of conditions for estimating specific activity must be somewhat arbitrary under such circumstances. In actual practice during fractionation, the conditions were sufficiently reliable to permit recovery of most of the initial activity. Muscle extract was routinely added in excess and, when necessary, 1 mg. of a partially purified arginase preparation, having a specific activity of 34 units per mg., was also added (see "Experimental").

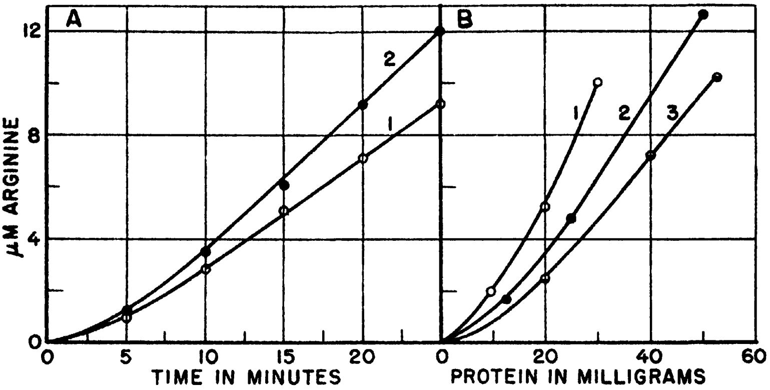

FIG. 2. Dependence of the rate of arginine synthesis on time and on enzyme concentration. In *A*, Curve 1 represents acetone powder extract containing 52 mg. of protein per tube; Curve 2, the same, supplemented with muscle extract. In *B*, Curves 1 and 2 represent alcohol-fractionated preparations of specific activity 1.0 and 0.75 respectively; Curve 3, acetone powder extract of specific activity 0.57. The conditions are otherwise as in Table I.

Data for the specific activity of several alcohol-precipitated fractions, as compared with the initial extract, are given in Table III. In the particular preparation recorded, after one fractionation procedure, the activity was found to concentrate in Fractions 3 and 4, the latter having the highest activity and a 3-fold purification over the original extract. Difficulty was occasionally encountered in reproducing these values because of the two-component nature of the system.

In the fifth column the activity is expressed in terms of the Q_{urea} value of Krebs to facilitate comparison of liver acetone powder extracts with homogenates under the conditions of Cohen and Hayano (2) and with slices under the original conditions of Krebs and Henseleit (1); *i.e.*, ornithine, NH_3, and lactate. The latter comparison is perhaps justified, since Gornall and Hunter (21) have shown that the conversion of citrulline to arginine is the rate-limiting step of the ornithine cycle.

Arginine Synthesis in Homogenates and Slices—On comparing the total activity of acetone powder extract of rat liver (calculated from the specific activity) to that of the tissue homogenate or slice, it appears that about 50 per cent of the original activity can immediately be accounted for. If allowance is made for some destruction by acetone treatment and for the fact that a single extraction might not be exhaustive, the result indicates that a large amount of the arginine-synthesizing activity is to be found in the isolated system. Although a discrepancy appears to exist between homogenates and the isolated system with respect to the specificity of the —NH_2 donor, the data in Table III leave little doubt that the same enzyme system is involved, regardless of the substrates offered or of other variations in the experimental conditions. The fact that in extracts aspartic acid can be replaced, through transamination, by the combination of glutamate and oxalacetate suggests that in homogenates glutamic acid functions in arginine synthesis indirectly, by transfer of the —NH_2 group to oxalacetate, rather than by an interaction with citrulline catalyzed by a second arginine-synthesizing system exhibiting glutamate specificity. Additional evidence for this and for the apparently poor activity of aspartic acid in liver homogenates will be discussed in Paper II.

TABLE III

Specific Activity of Various Liver Preparations

Species	Preparation	Conditions	Specific activity	Q_{urea}
Rat	Slice	NH_3, lactate[*]		18
"	Homogenate	Glutamate[†]		14
"	"	Aspartate, pyruvate[‡]		16
"	Acetone powder extract	" ~ph	0.84	18.8
"	" " "	" " + muscle extract	0.90	20.2
Ox	" " "	" "	0.33	7.4
"	" " "	" " + muscle extract	0.50	11.2
"	EthOH Fraction 3	" "	0.35	7.8
"	" " 3	" " + muscle extract	0.89	20.0
"	" " 4	" "	0.80	17.9
"	" " 4	" " + muscle extract	1.50	33.6

[*] Q_{urea} (microliters per mg. of protein per hour) recalculated from the data of Krebs and Henseleit, assuming 85 per cent of the dry weight to be protein.

[†] Calculated from the rate of arginine synthesis per mg. of N given by Cohen and Hayano.

[‡] Calculated from the data given in Table I of Paper II, assuming 85 per cent of the dry weight to be protein.

In both liver slices and homogenates, urea synthesis has been reported to be completely dependent upon a supply of oxygen. In Step II of the ornithine cycle, any direct oxidation-reduction mechanism has been excluded, but the participation of ~ph has been shown to be essential for arginine synthesis. The oxygen dependence in respiring preparations must then be associated with the generation of ATP by oxidation of respiratory substrates. In slices, added lactate, and in homogenates some of the glutamic acid serves in this capacity. The coupling of phosphorylation with the oxidative steps of the tricarboxylic cycle has been observed repeatedly in tissue preparations (22).

EXPERIMENTAL

Methods—The enzymatic reaction was carried out in small test-tubes kept at 0° prior to incubation. All additions were made with chilled solutions of substrates, previously adjusted to pH 7.5 with dilute KOH; the cold enzyme solution was added last. The tubes were then transferred to a water bath at 38° for the stated time interval, allowing 2 minutes for temperature equilibration, and the reaction was stopped with 2 ml. of 15 per cent metaphosphoric acid per 4 ml. of reaction mixture. Water was then added to a volume of 10 ml., the mixture filtered, and 0.5 ml. aliquots of the filtrate used for the estimation of urea by the method of Archibald (14) modified slightly. When more precise temperature control was necessary, all the additions except ATP were made at 0°. The tubes were then set in the water bath and the ATP added after temperature equilibration. This procedure gave slightly lower values. The complete system, as used routinely for measurements of enzyme activity, contained, in addition to the enzyme in 0.05 M potassium phosphate buffer at pH 7.5, 0.25 M potassium phosphate buffer, 0.4 ml.; 0.1 M L-aspartate, 0.2 ml.; 0.1 M L-citrulline, 0.2 ml.; 0.033 M $MgSO_4$, 0.4 ml.; 0.05 M ATP, 0.08 ml.; 0.1 M 3-phosphoglyceric acid, 0.5 ml.; when indicated, 8.8 mg. of muscle fraction in water; and water to make a final volume of 4 ml. To conserve materials, reactions were frequently carried out on half the scale and the analytical data doubled to corresponding full scale values. The amount of arginine formed was calculated from the urea estimation, since arginase was always present in excess.

When malic acid was also estimated, 0.25 M glycylglycine buffer replaced phosphate. The reaction was stopped by heating the tubes in a boiling water bath for 10 minutes after bringing the mixture to pH 6.0 with 0.02 to 0.04 ml. of 1 N sulfuric acid, the tubes were cooled to room temperature, water was added to a volume of 10 ml., and the mixture filtered. Malate was estimated spectrophotometrically in suitable aliquots of the filtrate by means of the "malic" enzyme of pigeon liver, as described by Ochoa, Mehler, and Kornberg (15).

Transaminase activity was estimated by the method of Green, Leloir, and Nocito (16) and is expressed in their units, one unit being that amount of enzyme which forms oxalacetic acid equivalent to 100 μl. of CO_2 in 10 minutes at 38° under given conditions.

Protein was estimated spectrophotometrically at 280 mμ, corrected for nucleic acid impurities according to Warburg and Christian (12).

Preparation and Fractionation of Enzyme System—Acetone powder was prepared in a cold room at 2° from fresh ox liver chilled at the slaughterhouse. Small trimmed pieces amounting to 115 gm. were ground for 30 seconds in the Waring blendor with about 200 ml. of acetone, previously chilled to −5°. The contents were rapidly transferred to a beaker with more acetone at −5° (10 volumes in all), stirred rapidly for 10 to 15 seconds, and filtered by suction on a large Büchner funnel. Rapid stirring with 10 volumes of acetone was repeated and the mixture was again filtered rapidly with suction. The tightly packed cake was rapidly spread out at room temperature with constant mixing to facilitate rapid drying. The activity of the dry powder stored at 2° slowly falls, decreasing to about half the value in 5 to 6 weeks.

The powder was extracted with 5 volumes of 0.1 M potassium phosphate buffer, pH 7.5, with mechanical stirring at room temperature for 20 minutes, centrifuged cold at 15,000 R.P.M., and the supernatant dialyzed at 2° against 0.05 M buffer overnight to insure zero blanks. These crude extracts contain approximately 75 mg. of protein per ml. after dialysis and, when stored at 2°, maintain activity for about 2 days but fall off rapidly thereafter.

Acetone powders of rat liver were made in a similar manner and may be stored for several weeks in the cold without loss of activity, but the extracts were found to be much more unstable than ox preparations. The extraction with 5 volumes of buffer was therefore carried out at 0° as well as centrifugation, and cold dialysis was limited to a 3 hour period. With these precautions the activity falls off to about 50 per cent in 24 hours and is almost completely gone 2 days after extraction.

In a typical alcohol fractionation, 315 ml. of buffered extract prepared from 100 gm. of beef liver acetone powder were chilled to 0°, transferred to a bath kept at −5°, and absolute ethyl alcohol, kept at −50°, added very slowly with mechanical stirring. The temperature of the mixture was not allowed to rise above 0° at the beginning of the addition and thereafter was allowed to drop slowly to −5° by the time the first alcohol addition was completed. The mixture was centrifuged at −5° for 1 hour at 3000 R.P.M. and the supernatant rapidly decanted into a beaker immersed in a −10° bath. The tubes containing the precipitate were immediately packed in cracked ice, and the precipitates taken up in a total of 10 to 20 ml. of 0.1 M buffer and dialyzed in 0.05 M buffer overnight to remove traces of alcohol.

Subsequent additions of alcohol to the supernatant were made in a similar manner, except that the temperature of the extract was maintained at −10° for both the alcohol addition and centrifugation. Four fractions were thus obtained by stepwise additions of 45, 25, 35, and 30 ml. of alcohol, corresponding to 12.5, 18.2, 25, and 30 per cent alcohol for each fraction. The alcohol concentration was calculated without correcting for the volume of the precipitate removed. The specific activity was 0.0, 0.40, 0.89, and 1.50, respectively. The total activity represented a 72 per cent recovery.

Isolation and Identification of l-Malic Acid—A large enzymatic run, on 25 times the scale described above, was carried out at 38° with an excess of alcohol-fractionated enzyme containing 1505 units (1.81 gm., specific activity 0.83, without added muscle extract). After 60 minutes, 26.4 mg. of urea (440 μM) were found, corresponding to 59 mg. of malic acid and 57.2 mg. of ornithine. The reaction mixture was acidified to pH 6.0 with 3.5 ml. of 1 N sulfuric acid, deproteinized by heating in a bath at 100°, the filtrate brought to pH 1.0 with 6 ml. of 6 N sulfuric acid, and extracted with ether in a continuous extractor for 72 hours. The ether layer was then taken to dryness, dissolved in water, filtered, and brought to a volume of 10 ml. Enzymatic analysis indicated that 38.6 mg. (65 per cent of theory) were recovered. After evaporating the solution to dryness, the malic acid was then converted to the cinchonine salt by refluxing in 5 ml. of acetone with 74 mg. of *d*-cinchonine for 30 minutes. After standing at 0° overnight, 78 mg. of crude salt were obtained. On three recrystallizations from methyl alcohol-acetone, and one from water-acetone, the melting point was 191–192° uncorrected. The mixed melting point, with an authentic sample of melting point 192°, was 191–192° uncorrected; the reported melting point was 198° (23). The optical rotation of the cinchonine salt was

$$[\alpha]_{\mathrm{D}}^{19^\circ} = +146^\circ\ (0.515\%\ \text{in water};\ l = 2.0)$$

The optical rotation of the uranium salt was determined under the general conditions of Dakin (23) adapted to small scale as follows: 2.5 ml. of the solution of cinchonine *l*-malate were quantitatively decomposed by the addition of 0.5 ml. of 0.14 N ammonia and filtered. To 2.0 ml. of the filtrate was added 0.5 ml. of 2.5 per cent uranium acetate, slightly acidified with acetic acid, and the solution was allowed to stand for 1 hour at room temperature.

$$[\alpha]_{\mathrm{D}}^{21} = -481^\circ\ (0.1076\%;\ l = 2.0)$$

The optical rotations reported by Dakin (23) were

$$[\alpha]_{\mathrm{D}}^{19^\circ} = +146^\circ\ \text{for cinchonine } l\text{-malate}$$

$$[\alpha]_{\mathrm{D}}^{19^\circ} = -482^\circ\ \text{for the uranium salt of } l\text{-malic acid}$$

Isolation and Identification of Ornithine—The aqueous layer (about 125 ml.) obtained after ether extraction was brought to pH 9 with a few ml. of 50 per cent NaOH, and benzoylated in the usual manner with 0.35 ml. of benzoyl chloride and 6 ml. of 1 N NaOH. The mixture was filtered at pH 7, acidified, and kept at 0° overnight. The crude crystalline dibenzoylornithine, obtained after filtering off the crystals and washing thoroughly with ether, weighed 133 mg. (88 per cent yield). On three recrystallizations from ethyl alcohol-acetone mixture, the compound melted at 186° uncorrected. $[\alpha]_D^{21} = +9.3°$ (3.60 per cent in 1 N NaOH) nitrogen (Kjeldahl); found 8.2 per cent, calculated 8.2 per cent.

Chemical Preparations—D-3-Phosphoglyceric acid was prepared by the method of Neuberg and Lustig (24). Fleischmann's commercial bakers' yeast,[4] dried in the laboratory, gave good yields with glucose and added DPN. ATP[5] was prepared according to Lohman (25). L-Citrulline was prepared by a combination of the methods of Kurtz (26) and Gornall and Hunter (27), starting with a commercial preparation of L-arginine. We are indebted to Dr. H. B. Dunn for DL-citrulline, and to Dr. H. Waelsch for DL-α-aminoadipic acid and DL-α-aminopimelic acid. The other amino acids were commercial preparations of acceptable purity. Oxalacetic acid was prepared according to Krampitz and Werkman (28), as modified by F. Lipmann (personal communication).

Other Enzyme Preparations—Partially purified arginase was prepared from a Mn^{++}-activated extract of beef liver by acetone fractionation according to Van Slyke and Archibald (29).[6] The arginase activity of the preparation was 34 units per mg. of protein when the unit and assay conditions of Van Slyke and Archibald (30) were employed, 1 unit being that amount which liberates 1 μM of arginine in 1 minute under given conditions.

The extract of rabbit muscle was prepared according to Racker (31). The fraction precipitating between 50 and 72 per cent ammonium sulfate saturation was dialyzed for 2 hours against running tap water and overnight against 0.02 M potassium phosphate buffer, pH 7.5, and then lyophilized. Stored at 2°, the dry powder retains activity for several months. Under the experimental conditions described, 8.8 mg. for each tube supplied an excess of the required activity. We are indebted to Dr. E. Racker for a gift of this fraction.

SUMMARY

1. An enzyme system catalyzing the conversion of citrulline to arginine has been isolated from mammalian liver and partially purified.

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[4] We are indebted to Mr. R. F. Light, the Fleischmann Laboratories, of Standard Brands, Incorporated, for a large gift of yeast.

[5] The ATP employed was 80 to 85 per cent pure. The amounts employed are given on a 100 per cent basis.

[6] Van Slyke, D. D., and Archibald, R. M., personal communication.

2. Evidence is presented to show that in the reaction L-citrulline and L-aspartic acid undergo an exchange to form L-arginine and *l*-malic acid through an intermediary condensation which utilizes ATP and Mg^{++}, and is presumably endergonic.

3. Two separate enzymes are involved in the over-all reaction, one catalyzing the formation of an intermediary condensation product and the second its hydrolysis.

4. Some general enzymatic properties of the system are described.

5. The mechanism is believed to represent the main physiological pathway of arginine synthesis and urea formation.

We are indebted to Mr. Morton C. Schneider for technical assistance.

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BIOSYNTHESIS OF UREA

II. ARGININE SYNTHESIS FROM CITRULLINE IN LIVER HOMOGENATES*

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A preceding paper described the characteristics of the isolated enzyme system, prepared from ox liver acetone powder, which catalyzes the conversion of citrulline and aspartic acid to arginine and malic acid. It was shown that the fundamental requirements for arginine synthesis from citrulline are aspartic acid, Mg^{++}, and adenosine triphosphate (ATP), the latter as a reactant in substrate concentrations (1). At the optimum enzymatic conditions established, a large proportion of the arginine-synthesizing activity of the tissue was found in the acetone powder extract.

Aspartic acid was shown to be the specific $—NH_2$ donor. Glutamic acid was unreactive but the combination of glutamic and oxalacetic acids could replace aspartic acid in proportion to the glutamic-aspartic transaminase activity of the enzyme preparation. The relative activity of these two amino acids is reversed in liver homogenates. Cohen and Hayano (2), corroborated by Krebs and Eggleston (3), have found that glutamic acid is about 4 times as effective as aspartic acid. Since these observations raise the question as to whether the enzyme system studied by us is the same as the one concerned with arginine synthesis in homogenates and slices, studies of liver homogenates bearing on this point were carried out. The experimental observations presented here show that the same enzymatic system is involved in all cases. It has been possible to explain conflicting observations by a uniform mechanism and to indicate the physiological pathway of amino nitrogen from amino acids to urea.

In order to relate the behavior of liver homogenates in oxygen to the behavior and requirements of the isolated system, certain general properties of homogenates, affecting arginine synthesis, should be mentioned. The adenosinetriphosphatase (ATPase) activity is much greater than that of acetone powder extracts; hence rapid generation of $\sim$ph is necessary. In addition to the enzyme system catalyzing the citrulline to arginine re-

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action, homogenates contain appreciable concentrations of several enzymes associated with glutamic acid metabolism; glutamic-aspartic transaminase, glutamic-alanine transaminase, and glutamic dehydrogenase. Associated with homogenate particles are all the respiratory enzymes and cofactors which, except for the additional requirement of adenylic acid or ATP and Mg^{++}, catalyze the coupled oxidative phosphorylation of respiratory substrates via the tricarboxylic cycle. The particles also contain the various factors involved in the transport of hydrogen to oxygen through pyridine nucleotides and the cytochrome system. Pyridine nucleotides and cytochrome need not be added, but if the latter becomes limiting, the whole tricarboxylic cycle shows cytochrome dependence. These are complexities contributed by the use of homogenates and are not necessarily inherent in the mechanism of arginine synthesis. Oxalacetic acid can be supplied by a rapidly respiring homogenate, since it lies in the pathway of the tricarboxylic cycle.

Generation of High Energy Phosphate—Under the conditions of Cohen and Hayano (2), ATP is supplied only in catalytic amounts, and no respiratory substrate other than the $—NH_2$ donor is added. It may be anticipated then that the ATP requirement must be met by oxidative phosphorylation and that the activity of the $—NH_2$ donor being tested would be limited by its capacity to act as a respiratory substrate. That such is actually the case may be shown by estimating the oxygen consumption of rat liver homogenates during arginine synthesis. L-Aspartic acid is very poorly, or not at all, oxidized by homogenates. As seen in Table I, in the presence of citrulline and aspartic acid, under aerobic conditions, the average arginine formation was 3.5 μM and the average oxygen consumption was 14.9 μM, a value approximately the same as the endogenous respiration of homogenates alone. The results were quite variable within these low limits and undoubtedly reflect the concentration of endogenous respiratory substrates at the time the tissue was obtained. With glutamic acid in place of aspartic acid, an average of 9.6 μM of arginine was formed and the oxygen consumption averaged 30.5 μM.

However, when a respiratory substrate such as pyruvic acid was added to aspartic acid, arginine was increased to 12.8 μM and the average oxygen uptake amounted to 29.4 μM. Similar accelerating effects on both arginine formation and oxygen uptake were observed when either phosphoglycerate, fumarate, oxalacetate, or α-ketoglutarate was added to aspartate (Table I). In homogenates under aerobic conditions, phosphoglycerate apparently behaved primarily as a source of pyruvate. This may be inferred from the fact that the average oxygen consumption was increased almost as much by the addition of phosphoglycerate as by that of pyruvate. The contribution of $\sim$ph from phosphopyruvate was probably of minor significance as compared to the amount generated by pyruvate oxidation. The acceleration of arginine formation from aspartic acid and citrulline by addition of the various substrates shown in Table I demonstrates that when aspartic acid is supplemented with a source of ATP, supplied by oxidative phosphorylation, arginine synthesis increases with the rate of ∼ph generation. This behavior is entirely consistent with the requirements outlined above and any member of the tricarboxylic cycle would be expected to have this accelerating action on arginine synthesis when aspartic acid is employed.

TABLE I

Synthesis of Arginine from Citrulline in Rat Liver Homogenates under Aerobic Conditions in Absence and Presence of Malonate

In addition each vessel contained 20 μM of L-citrulline, 5 μM of ATP, 10 μM of $MgSO_4$, 0.3 ml. of 0.25 M potassium phosphate, pH 7.5, and 0.5 ml. of 25 per cent homogenate. Final volume 3.0 ml.; 38°; time 40 minutes. The values are given in micromoles.

Substrate added, 20 μM	With L-aspartate				With L-glutamate			
	Arginine		O_2 uptake		Arginine		O_2 uptake	
	Average	A.D.[*]	Average	A.D.	Average	A.D.	Average	A.D.
None	3.5	±0.8	14.9	±0.2	9.6	±1.1	30.5	±2.7
Pyruvate[†]	12.8	±0.7	29.4	±1.2	6.0	±1.0	30.5	±1.8
3-Phosphoglycerate	12.9	±0.9	28.9	±1.2	6.2	±1.1	22.1	±5.5
Oxalacetate	14.4	±0.4	30.1	±1.9	10.9	±1.2	28.8	±5.0
Fumarate	13.4	±1.0	27.4	±1.6	12.5	±0.8	30.8	±2.4
α-Ketoglutarate	11.1	±0.4	23.7	±3.9	4.4	±0.7	23.1	±3.1
With 60 μM malonate per vessel								
None	3.3	±1.0	10.8	±2.0	2.6	±0.0	18.9	±0.6
Pyruvate	11.4	±0.2	19.9	±1.3	2.6	±0.4	19.1	±1.4
3-Phosphoglycerate	11.8	±1.3	21.5	±2.3	2.3	±0.3	17.7	±2.0
Oxalacetate	13.3	±0.4	24.1	±1.9	13.3	±0.3	27.4	±2.6
Fumarate	13.3	±0.5	25.4	±1.6	12.8	±1.0	28.1	±3.7
α-Ketoglutarate	11.8	±0.1	19.8	±2.7	1.5	±0.1	13.8	±1.9

[*] Average deviation.

[†] 2.5 μM of fumaric acid were added as a primer. Good respiration was often obtained without it, but the addition insured uniformly high values. Fumarate was not added to pyruvate when glutamate was employed; nor was it added in any malonate experiment.

Multiple Function of Glutamic Acid—When the same substrates were added to glutamic acid, different effects were observed, as shown in Table I. The addition of either pyruvate or phosphoglycerate caused a 40 per cent inhibition of arginine synthesis and a variable decrease in oxygen consumption. The addition of oxalacetate or of fumarate caused a slight increase in arginine synthesis without appreciably affecting the average oxygen consumption, while the addition of α-ketoglutarate caused a decrease in both arginine formation and oxygen uptake.

The high activity of glutamic dehydrogenase in liver homogenates accounts for the fact that glutamic acid is as good a respiratory substrate as α-ketoglutaric acid (4). Furthermore only a fraction of the glutamic acid present need be oxidized to furnish a large supply of ATP, for, as shown by Ochoa, the oxidation of α-ketoglutarate to succinate generates three $\sim$ph bonds (5) and further oxidation to fumarate generates one such bond, while the further complete oxidation of pyruvate is associated with the generation of fifteen $\sim$ph bonds (6). The dependence of α-ketoglutarate oxidation upon cocarboxylase (5–7) is therefore related to the decreased arginine synthesis observed in livers of vitamin B_1-deficient rats (8) by von Fahrländer, Nielsen, and Leuthardt.

If the point of view is taken that glutamic acid is active in so far as it can be converted to aspartic acid, once the $\sim$ph requirement is satisfied, then the acceleration of arginine synthesis by addition of oxalacetate to glutamate[1] may be explained as being primarily due to the appearance of extra aspartic acid formed by transamination with glutamic acid. Fumarate, being an immediate precursor of oxalacetate, had the same effect. Malate would be expected to act similarly. In the absence of added oxalacetate, aspartate can arise from glutamate by transamination with the oxalacetate formed during oxidation of glutamate via α-ketoglutarate and the Krebs tricarboxylic cycle. Oxalacetate will also be formed by a one-step oxidation of the malic acid appearing as the second product in arginine synthesis.

Although the synthesis of aspartic acid has not been shown directly in homogenates when glutamic acid is the substrate offered, much indirect evidence indicates that such must the case. No satisfactory explanation has yet been offered of the inhibition, reported from other laboratories, of arginine synthesis by pyruvate (2, 8, 9), by α-ketoglutarate (3, 8, 9), and malonate (2, 3, 10) when glutamate is the $-NH_2$ donor. These inhibitions may all be explained as being due to an interference either with the formation of or with the further reaction of aspartic acid.

Inhibition by α-Ketoglutarate—Cohen has reported the various transaminase activities of rat liver. From his data (11) and from what is now known of the relative activities of glutamic-aspartic transaminase and of glutamic-alanine transaminase (12, 13), the rates of the three transamination reactions listed in descending order of activity are glutamate-oxalacetate, glutamate-pyruvate, and aspartate-pyruvate, the first being by far the most rapid and the third rather slow, since it is the combined result of the first two reactions (12, 14).

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[1] When the rate of arginine synthesis is decreased by reducing the ATP concentration, a 30 to 40 per cent stimulation by oxalacetate can be demonstrated.

Inhibition of arginine synthesis by α-ketoglutaric acid when glutamate is the source of $—NH_2$ varies from about 40 to 60 per cent, depending on the amount of keto acid added (3, 9, 15). As shown in Table I, with 20 μM each of α-ketoglutarate and glutamate, a 54 per cent inhibition was found. This inhibition may be attributed to reversal of the transamination between glutamate and oxalacetate; *i.e.*, glutamate + oxalacetate $\leftrightarrows$ α-ketoglutarate + aspartate. Such inhibition would not be expected if glutamic acid were the specific $—NH_2$ donor. The inhibition by α-ketoglutarate would furthermore tend to be maintained by slow oxidative removal from a reaction mixture already containing enough keto acid (derived from the glutamic acid) to saturate the tricarboxylic cycle.

On the other hand, with 20 μM each of α-ketoglutarate and aspartate, a stimulation of arginine synthesis was observed almost as great as that produced by the addition of other respiratory substrates. Here the aspartate concentration is initially in excess as far as enzyme saturation is concerned. The addition of an equal amount of α-ketoglutarate might at most reduce the aspartate to 15 μM by transamination, but even at this level the rate of arginine synthesis would be expected to be quite rapid providing ATP were being supplied, as it is in this case, by α-ketoglutarate oxidation. Under these conditions the stimulation caused by supplying ATP is the main effect observed.

Von Fahrländer, Nielsen, and Leuthardt (8) have shown that the α-ketoglutarate inhibition can be overcome by added NH_3. The observation is consistent with the explanation given above, for the addition of NH_3 to α-ketoglutaric acid would remove some α-ketoglutaric acid by reductive amination to glutamic acid.

Inhibition by Pyruvic Acid—As shown in Table I, pyruvate stimulates arginine formation when aspartate is the source of $—NH_2$, but causes a 40 per cent inhibition when glutamate is the $—NH_2$ donor. This inhibition can also be explained as an effect on the formation of aspartic acid caused by the removal of some glutamic acid from the reaction mixture by transamination, even though glutamic-pyruvic transamination is relatively slow in liver. The appearance of α-ketoglutaric acid, formed by the transamination of glutamate with pyruvic acid, would augment the inhibition, as explained in the preceding section.

As with α-ketoglutarate, the pyruvate inhibition is also relieved by added NH_3 (8), an effect which may be explained in the same way. The similar results obtained with phosphoglyceric acid (Table I) are in accord with the rapid conversion of the latter to pyruvic acid.

Krebs and Eggleston (3) reported an acceleration of the synthesis of arginine by citrate and succinate when aspartate is the source of $—NH_2$. This is understandable in so far as oxidation of these substrates through the tricarboxylic cycle generates the necessary ~ph. Inhibition by citrate when glutamate is the source of $—NH_2$ (3) may be explained by oxidation of citrate to α-ketoglutarate. The inhibition by succinate (3) observed under these conditions, however, is not easy to understand unless it is assumed that succinate interferes with the oxidation of α-ketoglutarate derived from glutamate.

Inhibition by Malonic Acid—The inhibition by malonate of arginine formation when glutamate is the source of $—NH_2$ has been reported by Cohen and Hayano (2) and confirmed in the laboratories of Leuthardt (10) and of Krebs (3). The latter two groups have also shown that fumarate overcomes the inhibition. A comparison of the data obtained without malonate with corresponding values obtained in the presence of malonate (Table I) again confirms these observations. This comparison shows further that the inhibition is not exerted on arginine synthesis *per se*. When aspartate was added to homogenates under optimum conditions, *i.e.* in the presence of a source of ATP, no malonate effect was observed. A satisfactory explanation of all these observations can be offered in terms of the specificity for aspartic acid in the conversion of citrulline to arginine and of the well known inhibition of succinic dehydrogenase by malonate (16). When oxidation of α-ketoglutarate is blocked at the succinate stage, oxalacetate will not be available for the synthesis of aspartic acid.

The main inhibitory effect on glutamate is clearly not associated with interference of ATP generation, for the addition of malonate to aspartate plus pyruvate only decreased arginine formation from an average of 12.8 μM to an average of 11.4, while lowering the oxygen uptake from an average of 29.4 μM to an average of 19.9. In the absence of malonate, respiration and ATP generation were presumably excessive, so that the appreciable reduction of oxygen consumption caused by malonate resulted only in a small decrease in arginine synthesis. Similarly with aspartic acid as the source of $—NH_2$, in the presence of either phosphoglycerate, oxalacetate, fumarate, or α-ketoglutarate, malonate caused but a small reduction of arginine synthesis along with an appreciable reduction in oxygen consumption. The oxidation of these substrates is of course curtailed at the succinate stage. The oxidation step, α-ketoglutarate to succinate, as mentioned above, yields three ~ph bonds per atom of oxygen. The high ratio explains why an adequate supply of ATP can be maintained in the presence of malonate. A high final concentration of malonate was employed (0.02 M) in order to assure a maximum inhibition of succinate oxidation.

In contrast to the results with aspartate, malonate caused a large decrease in arginine synthesis when glutamate was the source of $—NH_2$. Oxygen consumption was appreciably reduced, as with aspartate. Thus, the oxygen fell from an average of 30.5 μM to 18.9, while the synthesis of arginine decreased from an average of 9.6 to 2.6 μM. As shown in Table I, oxalacetate overcomes the malonate inhibition. Fumarate had a similar effect, obviously due to its conversion to oxalacetate by oxidation. Malate would be expected to have the same effect. Pyruvate, phosphoglycerate, and α-ketoglutarate, which in the presence of malonate continued to produce a stimulation of arginine synthesis, with aspartate as the source of $—NH_2$, had no effect in overcoming malonate inhibition of glutamate, since they cannot supply oxalacetate in the presence of malonate and in addition are inhibitory by themselves.

TABLE II

Synthesis of Arginine from Citrulline in Rat Liver Homogenates under Anaerobic Conditions

Further additions and other conditions are as in Table I, except that N_2 replaced O_2 in the gas space and 4 mg. of DPN were added to each vessel containing hexose diphosphate. The dry weight of 0.5 ml. of homogenate and 0.4 ml. of supernatant respectively was 34.7 mg. and 17.1 mg. The values are given in micromoles.

Substrate added					Arginine found	
L-Aspartate, 20 μM	L-Glutamate, 20 μM	Oxalacetate, 30 μM	3-Phosphoglycerate, 40 μM	Hexose diphosphate, 25 μM	Whole homogenate	Supernatant
+			+		3.9	7.7
+				+	5.7	8.0
+					0.6	1.2
	+		+		0.1	0.0
	+			+	0.5	0.4
	+	+	+		4.6	6.8
	+	+		+	4.2	4.9

Anaerobic Synthesis of Arginine in Homogenates—Once the requirements of the system are known, it becomes possible to obtain arginine formation in homogenates under anaerobic conditions by utilizing glycolytic reactions to generate ~ph. Table II shows the amount of arginine formed in a representative experiment, in the presence of citrulline, aspartic acid, and phosphoglyceric acid or hexose diphosphate.[2] None was formed in the absence of a supply of ~ph. The addition of phosphoglyceric acid or hexose diphosphate caused no arginine formation when glutamic acid was substituted for aspartic acid, unless oxalacetic acid was also added. These results are similar to those obtained with acetone powder extracts. No further efforts were made to increase the rate of synthesis by improved conditions.

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[2] DPN was included with hexose diphosphate, since the anaerobic inactivation of DPN is very rapid. A dismutation between pyruvic acid and triose phosphate was undoubtedly responsible for the availability of ~ph.

The rates of arginine synthesis, anaerobically, were much lower, however, than those obtained with an equal amount of homogenate under aerobic conditions. Owing to the fact that the efficiency of anaerobic phosphorylation is lower than that of aerobic phosphorylation, competition with ATPase is less successful anaerobically. A considerable portion of the ATPase present is associated with the suspended particles of tissue homogenates and can be removed by high speed centrifugation. The last column of Table II gives the amounts of arginine synthesized by an equivalent amount of supernatant after removing the particles. Arginine, when formed, was found to be higher than in the whole homogenate in each case.

As a result of aerobic experiments with glutamic acid in which the supernatant and centrifuged particles of homogenates were studied separately, Cohen and Hayano (17) came to the conclusion that both the particles and the supernatant were essential for arginine synthesis. From the anaerobic data of Table II, it may be seen that both the arginine-synthesizing system and transaminase are present in the supernatant. It is clear from the foregoing discussion that the particles were required for the generation of ATP and of oxalacetic acid.

Since the behavior of liver homogenates, under a variety of experimental conditions, is in accord with predictions based upon the mechanism and requirements of the isolated system, there appears to be no need for considering that more than one enzyme system is concerned with the conversion of citrulline to arginine in mammalian liver. The evidence indicates that in homogenates glutamic acid must function as a nitrogen carrier, as ATP generator, and as a source of oxalacetic acid.

Physiological Pathway of Amino Nitrogen Transfer

The individual steps in the transfer of nitrogen from amino acids to form urea and their relationship to the tricarboxylic cycle are summarized in Fig. 1. In homogenates, when glutamic acid is the substrate offered, the transfer would start at Step B (transaminase) as soon as an adequate concentration of oxalacetic acid accumulates. In slices, when NH_3 and lactate are the substrates offered, nitrogen transfer to citrulline would start at Step A (glutamic dehydrogenase) with α-ketoglutaric acid made available from endogenous sources or from lactic acid oxidation. Hydrogen transport for Step A might come from malate oxidation or from other dismutations. The transfer of nitrogen to ornithine (Step E) is provisionally shown as occurring through NH_3. This step has been studied by Cohen and his collaborators. According to their most recent report (18) carbamylglutamic acid will replace the CO_2 but not the NH_3 requirement in citrulline formation from ornithine. In the intact animal NH_3, arising from oxidative deamination of amino acids (19–21), would enter, as with slices, at Steps A and E. The scheme represents an expansion of the ornithine cycle originally proposed by Krebs and Henseleit (22).

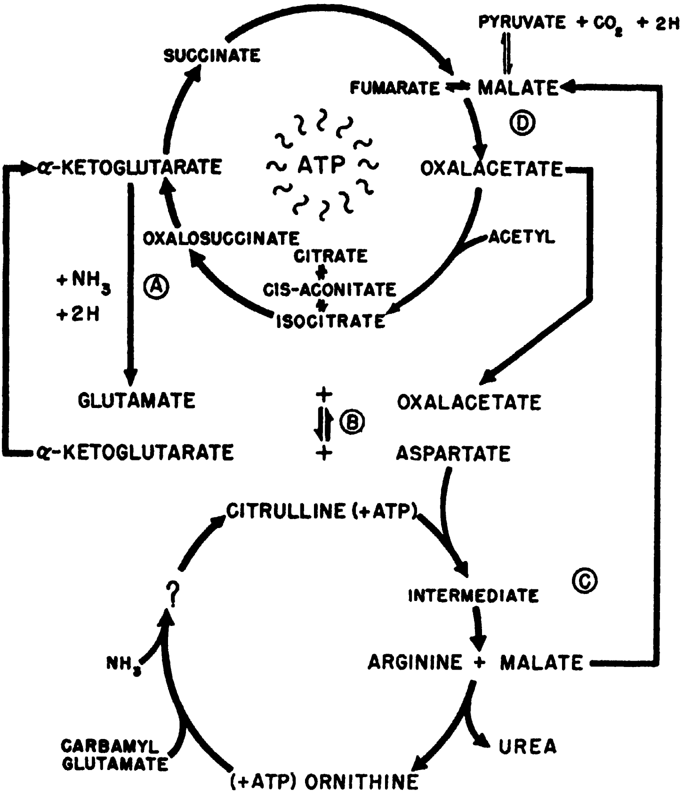

Fig. 1. Pathway of amino nitrogen transfer to citrulline in urea synthesis and the relationships of the ornithine cycle to the tricarboxylic cycle.

The interrelationships with the tricarboxylic cycle occur through several additional connecting cycles. The transfer of NH_3 to oxalacetic acid, through Steps A and B, is a cyclic process in which α-ketoglutaric acid, supplied from the tricarboxylic cycle, is utilized catalytically. Another cycle is created by the turnover of oxalacetic acid through Steps B, C (arginine synthesis), and D (malic dehydrogenase). At the same time phosphorylations, coupled with the tricarboxylic cycle, supply ATP for both citrulline and arginine synthesis.

Transfer of Amino Nitrogen to Oxalacetic Acid—The assumption of an obligatory incorporation of NH_3 in aspartic acid by transamination between glutamate and oxalacetate prior to urea formation (Steps A and B) appears to be justified for a number of reasons. Evidence is lacking for the existence in mammalian tissues of an aspartase or of an aspartic dehydrogenase analogous to glutamic dehydrogenase with respect to activity and reversibility. Also there is the observation that liver homogenates are incapable of oxidizing L-aspartic acid at an appreciable rate. In addition liver homogenates behave as though aspartic acid were an intermediate in arginine synthesis under a wide variety of conditions, as shown above. It may be pointed out that investigations of intact animals with N^{15}-labeled amino acids and NH_3 show that aspartic acid as well as glutamic acid have the highest rate of turnover (20). Finally, Steps A, B, C, and D can be carried out anaerobically in crude extracts of ox liver acetone powder which contain these enzymes, through the coupling of several oxidation-reductions with transamination.

Amino Nitrogen Transfer Anaerobically—In the first dismutation, the following reactions occur:

(1) Malic acid + $DPN_{ox.}$ $\leftrightarrows$ oxalacetic acid + $DPN_{red.}$

(2) NH_3 + α-ketoglutaric acid + $DPN_{red.}$ $\leftrightarrows$ glutamic acid + $DPN_{ox.}$

(3) Glutamic acid + oxalacetic acid $\leftrightarrows$ α-ketoglutaric acid + aspartic acid

(4) Malic acid + NH_3 $\leftrightarrows$ aspartic acid

The dismutation, catalyzed by malic and glutamic dehydrogenases in the presence of transaminase (Reactions 1, 2, and 3), resulted in the formation of aspartic acid (Reaction 4) starting with malate, NH_3, and α-ketoglutarate in the presence of catalytic amounts of diphosphopyridine nucleotide (DPN). Citrulline, ATP, Mg^{++}, and phosphoglyceric acid were also added, and the appearance of aspartic acid was measured by arginine synthesis, as shown in Fig. 2, Curve 1. Curve 1*a* represents blank values when either DPN, NH_3, or malic acid was omitted.

In the second dismutation, triose phosphate dehydrogenase (Reaction 5) replaced malic dehydrogenase.

(5) Triose phosphate + H_3PO_4 + $DPN_{ox.}$ $\leftrightarrows$ diphosphoglyceric acid + $DPN_{red.}$

(2) NH_3 + α-ketoglutaric acid + $DPN_{red.}$ $\leftrightarrows$ glutamic acid + $DPN_{ox.}$

(3) Glutamic acid + oxalacetic acid $\leftrightarrows$ α-ketoglutaric acid + aspartic acid

(6) Triose phosphate + H_3PO_4 + NH_3 + oxalacetic acid $\leftrightarrows$
diphosphoglyceric acid + aspartic acid

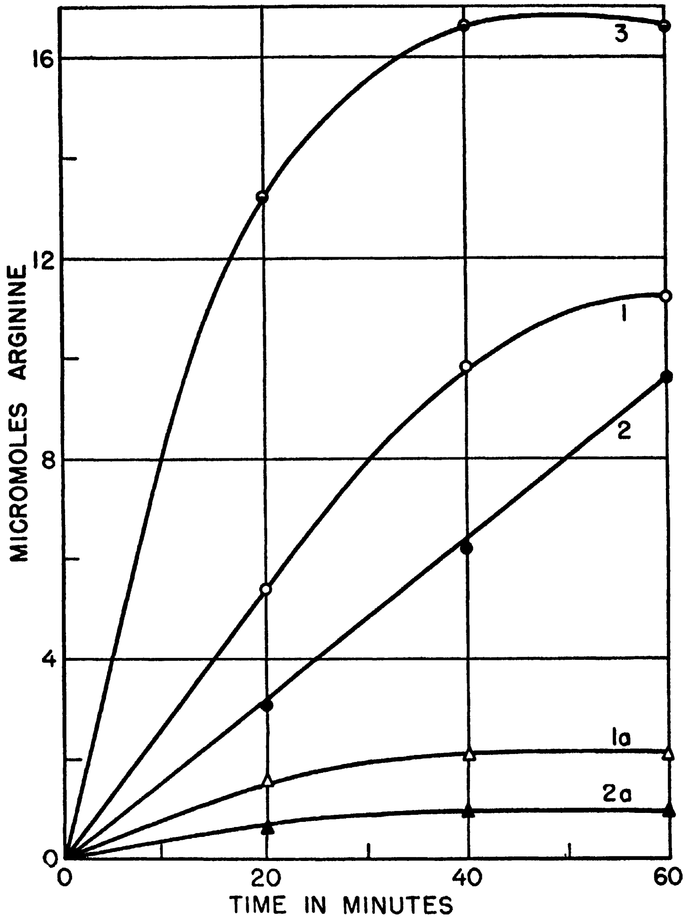

FIG. 2. Transfer of NH_3 to citrulline through aspartic acid by anaerobic dismutation and transamination in acetone powder extracts of ox liver. Curve 1, malate, α-ketoglutarate dismutation; Curve 1*a*, the same as Curve 1 in the absence of either DPN, NH_3, or malate; Curve 2, triose phosphate, α-ketoglutarate dismutation; Curve 2*a*, the same as Curve 2 in the absence of either DPN, NH_3, or oxalacetate; Curve 3, arginine formation with aspartate in the same amount of extract. All the tubes contained 4 μM of ATP, 20 μM of L-citrulline, 13 μM of $MgSO_4$, 0.4 ml. of 0.25 M potassium phosphate, pH 7.5, and 1.0 ml. acetone powder extract in a final volume of 4 ml.; temperature 38°. Further additions were Curve 1, 20 μM each of NH_4Cl and *l*-malate, 10 μM of α-ketoglutarate, 50 μM of D-3-phosphoglyceric acid, and 2 mg. of DPN. Curve 2, 20 μM of NH_4Cl, 10 μM of α-ketoglutarate, 30 μM of oxalacetate, 25 μM of hexose diphosphate, and 2 mg. of DPN. Curve 3, 20 μM of L-aspartate, 50 μM of 3-phosphoglycerate.

Here aspartic acid, as measured by arginine synthesis, was formed (Reaction 6) when oxalacetic acid, NH_3, hexose diphosphate,[3] DPN, α-ketoglutaric acid, ATP, Mg^{++}, and citrulline were added. This is shown in Curve 2. The diphosphoglyceric acid formed supplied ~ph. Curve 2*a* represents blank values when either DPN, NH_3, or oxalacetate was omitted. Curve 3 represents the rate of arginine synthesis in the same amount of extract starting with aspartic acid, ATP, Mg^{++}, and phosphoglyceric acid.

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[3] The extract contains aldolase.

One or more of the enzymes catalyzing Reactions 1, 2, 3, and 5 were probably present in limiting concentrations. Because of this and of the relatively high Michaelis constant of the arginine-synthesizing system with respect to aspartic acid (1.2×10^{-3} M), the rate of arginine synthesis was lower in Curves 1 and 2 than in Curve 3. On the other hand, because of the rather large Michaelis constant, the rate of aspartic acid synthesis in Curves 1 and 2 was doubtless somewhat higher than that reflected by the arginine values.

EXPERIMENTAL

Procedures—For the experiments with homogenates, the liver from young adult, well nourished rats was removed immediately after exsanguination, chilled 20 minutes in buffer, rapidly weighed, homogenized with 3 volumes of 0.1 M potassium phosphate buffer, pH 7.5, in a loose fitting Potter-Elvehjem homogenizer (23), and strained through two layers of cheese-cloth. All manipulations were carried out at 2°. Incubation was carried out at 38° in conical Warburg vessels containing a KOH solution in the center well. Prior to incubation the substrates (adjusted to pH 7.5) were added to the main compartment, the vessels immersed in an ice bath, and 0.5 ml. of the cold 25 per cent homogenate added last in a final volume of 3.0 ml. The vessels were gassed for 2½ minutes with O_2. The oxygen consumption was estimated for 40 minutes, following a 5 minute equilibration period. The amount of arginine synthesized corresponds to a slightly longer period. The reaction was stopped by adding 2 ml. of 15 per cent metaphosphoric acid. Urea estimations were carried out by the method of Archibald (24), with slight modification, and the values expressed as equivalent amounts of arginine. Arginase was present in excess. All values were corrected for the zero time urea content of the tissue and were then recalculated for a uniform dry weight of 31 mg.[4] They are so presented in Table I. Each value, representing the average of from three to five experiments, is given with the average deviation. Estimations based on Kjeldahl nitrogen indicated that 85 per cent of the dry weight of the homogenate was protein.

The anaerobic experiments were carried out in a similar fashion, except that the vessels were gassed with N_2. When the supernatant was to be used, the particles were separated by centrifuging for 30 minutes at 15,000

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[4] This value was chosen, since it represents the average dry weight of all the experimental samples.

R.P.M. at 0°. The sediment occupied 20 per cent of the total volume. No special precautions were taken to remove traces of oxygen in view of the order of magnitude involved.

The dismutation experiments were carried out with acetone powder extracts of ox liver freshly prepared as described previously (1). The specific activity, without added muscle extract, was 0.53.

Chemical Preparations—For the preparation of L-citrulline, D-3-phosphoglyceric acid, oxalacetic acid, and ATP, consult the preceding paper (1). The DPN employed was 75 per cent pure; it was prepared by a modification of the method of Williamson and Green (25).[5] α-Ketoglutaric acid was prepared by the method of Neuberg and Ringer (26).

SUMMARY

1. The synthesis of arginine from citrulline has been studied in liver homogenates under aerobic and anaerobic conditions, comparing aspartic acid with glutamic acid as $-NH_2$ donors.

2. Aerobically, arginine formation proceeds more rapidly with aspartic acid as $-NH_2$ donor than with glutamic acid, when the reaction mixture is supplemented with a respiratory substrate as a source of $\sim$ph.

3. Evidence is presented to show that oxidation of glutamic acid, through the tricarboxylic cycle, supplies both the aspartic acid and the $\sim$ph required for arginine synthesis. Under these conditions aspartic acid is formed by transamination of glutamic acid with the oxalacetic acid arising by glutamate oxidation. Energy-rich phosphate is generated by phosphorylations coupled with oxidation.

4. The inhibition of arginine synthesis by α-ketoglutarate and by pyruvate when glutamate is the $-NH_2$ donor is explained as being due to interference, at the transamination step, with the obligatory formation of aspartic acid. The inhibitions were not observed when aspartic acid was supplied directly.

5. Malonate has no effect on arginine synthesis *per se;* the inhibition observed when glutamate is employed is exerted through inhibition of succinic dehydrogenase, thus preventing oxalacetate formation. Oxalacetate or a closely related precursor therefore overcomes the inhibition.

6. Conditions for effecting the anaerobic synthesis of arginine in liver homogenates are described.

7. A scheme is presented for the physiological pathway of amino nitrogen transfer from amino acids to form urea, showing the interrelationships with the tricarboxylic cycle and with transamination.

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[5] We are greatly indebted to Mr. R. F. Light, the Fleischmann Laboratories of Standard Brands, Incorporated, for a large gift of yeast.

8. The anaerobic transfer of NH_3 to citrulline to form arginine has been carried out in acetone powder extracts of liver.

We are indebted to Mr. Morton C. Schneider for technical assistance.

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MUCOLYTIC ENZYME SYSTEMS

VII. EFFECTS OF TISSUE EXTRACTS AND BODY FLUIDS, CERTAIN STEROIDS, AND HEMOGLOBIN DERIVATIVES ON HYALURONIDASE ACTIVITY*

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The rôle of hyaluronidase as a spreading factor or invasive agent in tissues has led to studies of its occurrence in virulent bacteria, snake venoms, bee sting extracts, and spermatozoa (1, 2). Its presence in malignant cells has not been conclusively established. The earlier work of Duran-Reynals and Stewart (3), and the more recent studies of McCutcheon and Coman (4), on malignant tissues, favored its occurrence in some instances.

It has been shown that antibodies elicited by hyaluronidase protein specifically inhibit the enzyme which served as the antigen (1). Thus, a testicular antihyaluronidase will inhibit the testicular enzyme but not streptococcal hyaluronidase, and vice versa. Blood sera from many species have been found to inhibit hyaluronidases from various sources in a non-specific manner, indicating that other inhibitors must be present independent of specific antibodies (5–7). Glick and Moore (8) recently demonstrated by electrophoretic separation at pH 8.6 that the non-specific inhibitory activity of human serum is associated primarily with the albumin. Goldberg and Haas (9) reported a separation of the inhibitor in hog serum into two components, but later[1] they found that the effect of one of the components could be duplicated by magnesium ions. It was shown by Baumberger and Fried (10) that magnesium potentiates the inhibiting property of serum.

Previous investigations in this laboratory revealed that significant increases in the hyaluronidase-inhibiting ability of serum developed in poliomyelitis (11), in various infectious diseases (12–14) of both bacterial and virus origins, and in cancer (15). A strain difference in lines of mice with high cancer incidence was also noted (16). The present investigation was

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[1] Goldberg, A., personal communication.

originally undertaken in an attempt to determine the anatomical origin of the inhibitory factor in the serum, and accordingly various tissues and body fluids were studied. Subsequently, it was observed that certain bile constituents possessed inhibitory properties, and the investigation was extended to include the effects of a variety of these constituents, hemoglobin derivatives, as well as certain steroid hormones and related compounds. The rabbit was chosen as the source of tissues and of some of the body fluids investigated because of the ready availability of the fresh normal material.

Methods

The rabbit tissues and fluids were obtained from animals killed by intravenous air injection. The tissues were removed immediately, washed off with physiological saline to remove superficial blood, ground thoroughly in a mortar with sand, and extracted with isotonic saline solution. The extraction was carried out at room temperature for about 2 hours, after which the material was stored in the frozen state until ready for use. Subsequently it was thawed, centrifuged, and the supernatant drawn off for the analysis. The amount of extract employed in each reaction mixture generally represented about 0.5 gm. (wet weight) of original tissue. In the case of smaller organs, the entire organs, or the pooled material from several, were used. Serum alone, and combined with tissue extracts, were subjected to the same treatment without alteration of the hyaluronidase-inhibiting property.

Hyaluronidase from bull testes and hyaluronic acid from human umbilical cords were obtained by procedures essentially the same as those previously described (11). In the preparation of hyaluronic acid it was found more convenient to dehydrate the ground cord thoroughly by four additional treatments of 2 hours each, with enough fresh acetone to cover the material each time. The excess acetone was finally removed by suction on a Büchner funnel, and when the material was dry it was ground in a Wiley mill to a powder which passed a 30 mesh sieve. In this state the material could be stored indefinitely at room temperature without appreciably affecting the subsequent yield of hyaluronic acid.

The procedure for extracting the hyaluronic acid from the dried cord preparation was altered as follows: To each 120 gm. of cord powder, 200 ml. of Hayem's solution and 600 ml. of water were added, and the mixture was agitated in a Waring blendor for 6 minutes each day for 2 days. Between treatments, the material was stored in the cold. The mixture was then centrifuged for 6 minutes at about 2000 R.P.M., and the supernatant fluid was removed. The residue was reextracted with an equal volume of thrice diluted Hayem's solution by agitation in the blendor for 6 minutes, placed in a refrigerator overnight, again agitated for 6 minutes in the blendor, and refrigerated overnight, and finally centrifuged. The sedimented material was extracted two more times in this fashion (until the supernatant fluids were no longer viscous). The combined supernatants were clarified in the Sharples supercentrifuge and allowed to stand overnight in the cold. The clarification in the Sharples apparatus was carried out four more times in the same fashion. The hyaluronic acid was precipitated with acetone from the final clear liquid, dried, and the substrate solution prepared from it as previously described (11). The yield of dry hyaluronic acid prepared in the preceding manner is about 8 per cent, based on dry cord powder. This yield can be increased by working up the fine precipitate which was discarded during the acetone precipitation of the hyaluronic acid from the clarified aqueous solution.

The viscosimetric method previously employed for the measurement of hyaluronidase activity and its inhibition (11) was used in the present investigation.

Calculation of Hyaluronidase Inhibition

In previous publications from this laboratory, the hyaluronidase inhibition was expressed by the term employed by Haas (7), $(R - R_0)/R_0$, which is a function of the fractional change in viscosity, where R_0 is the time in seconds for the "relative viscosity" (η) of the reaction mixture to fall to one-half its initial value, and R is the corresponding time for η to fall to half in the presence of inhibitor. $\eta = (t_1 - t_2)/t_2$, where t_1 is the viscosity tube outflow time for the complete reaction mixture, and t_2 the corresponding time for the mixture without substrate present. The reaction time (t_3) that applies to a given measurement of η was taken as $t + (t_1/2)$, where t is the time from the original mixing of enzyme and substrate to the beginning of the outflow measurement.

Dorfman *et al.* (17) emphasized the advantage of expressing inhibition as $(1/R_0) - (1/R)$, a term representing the loss in enzyme activity. At 100 per cent inhibition, $(R - R_0)/R_0$ equals infinity, while $(1/R_0) - (1/R)$ equals $1/R_0$. There would be some advantage in employing the terms more commonly used in enzyme work, *viz.* per cent inhibition. Haas (7) actually did use this term in some cases, and he calculated it from the expression, $(100A/(A + 1))$, where $A = (R - R_0)/R_0$. When the term employed by Dorfman *et al.* (17) is used, the per cent inhibition would be $(100\ (1/R_0 - 1/R))/(1/R_0)$. By algebraic simplification, both terms for the per cent inhibition can be converted to $(100(R - R_0))/R$. The relation between per cent inhibition and A calculated from the same data obtained in an actual experiment is illustrated in Fig. 1. It will be seen that an approximately linear relationship obtains for lower inhibitions in both cases.

When dealing with tissue extracts or other liquids whose viscosities might significantly affect that of the reaction mixture, a correction factor is required. In attempting to work out this factor, it was observed that the magnitude of the viscosity increase occasioned by tissue extracts, or compounds which increase viscosity such as glycerol and gelatin, became progressively less as the hyaluronic acid was depolymerized. The difference in t_1 (ΔT) between hyaluronic acid solutions, with and without materials that increase the viscosity, was found to be considerably greater

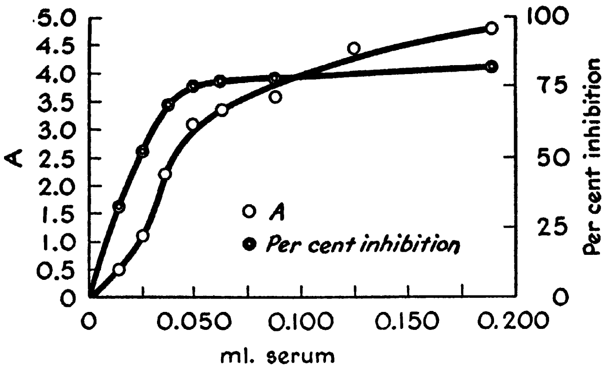

FIG. 1. Effect of quantity of human serum on hyaluronidase inhibition, expressed as per cent inhibition, and as A.

TABLE I

Values of F for Rabbit Tissue Extracts, Glycerol, and Gelatin*

Tissue	Amount of tissue in extract	ΔT_e	ΔT_o	F
	gm.	*sec.*	*sec.*	
Liver	1.6	28.5	9.0	0.66
Stomach	0.7	10.3	3.8	0.63
Small intestine	0.6	8.4	3.3	0.69
Spleen	0.4	4.0	1.8	0.72
Lung	0.8	7.0	3.5	0.76
Kidney	0.9	8.5	4.0	0.74
Average				0.70
Glycerol	0.3†	11.1	5.3	0.74
Gelatin	0.03	13.8	6.8	0.73

* A factor to correct for increased viscosity resulting from the presence of tissue extracts, etc.

† Ml.
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than that (ΔT_c) between corresponding control solutions in which the hyaluronic acid is omitted. In other words, the hyaluronic acid causes the added materials to increase the viscosity more than they would in the absence of the hyaluronic acid. To correct the value of R for the increased viscosity resulting from the presence of tissue extracts, etc., a factor (F) was employed which was obtained as follows:

With decreasing concentrations of hyaluronic acid, the value of ΔT approaches that of ΔT_c. It has been assumed that a linear relationship obtains between the decrease in viscosity due to depolymerization of hyaluronic acid and the accompanying fall in the value of ΔT. Hence, when η falls to half of its original value, the change in ΔT from zero time (ΔT_0) to time R can be considered $(\Delta T_0 - \Delta T_c)/2$, and the value of ΔT at time R becomes $\Delta T_0 - (\Delta T_0 - \Delta T_c)/2$. The proportion of this latter value to that at zero time is taken as the factor (F), or

TABLE II

*Effect of Concentration of Rabbit Intestinal Extract on Value of F**

Extract	ΔT_0	ΔT_c	F
ml.	*sec.*	*sec.*	
0.3	3.4	1.2	0.68
0.6	8.4	3.3	0.69
1.0	16.1	5.8	0.68
1.5	29.0	9.8	0.67

* A factor to correct for increased viscosity resulting from the presence of tissue extracts.

$$F = \frac{\Delta T_0 - \dfrac{\Delta T_0 - \Delta T_c}{2}}{\Delta T_0}$$

The relative constancy of F is shown in Table I for a group of representative tissues, glycerol, and gelatin. The effect of varying the concentration of a given tissue extract on the value of F is presented in Table II. A value of 0.7 for F has been used for the tissue experiments. The data from which ΔT_0 was calculated were obtained by repeated measurements of outflow times over a period approximately equal to that used in the actual inhibition determination. This also served as a control for the detection of the possible presence of hyaluronidase or any other substance in the tissue extract that might reduce the viscosity of the mixture during the course of the reaction.

The value of the "relative viscosity" at zero time of the reaction mixture containing enzyme, substrate, and the added solution (tissue extract, etc.), η_0, is given by

$$\eta_0 = \frac{t_{1_0} - \Delta T_0}{t_2} - 1$$

and when the "relative viscosity" falls to half of its original value due to depolymerization of hyaluronic acid

$$\frac{\eta_0}{2} = \frac{t_1 - 0.7\Delta T_0}{t_2} - 1$$

On rearranging the equation,

$$\frac{t_1}{t_2} - 1 = \frac{\eta_0}{2} + \frac{0.7\Delta T_0}{t_2}$$

Thus, by adding $(0.7\Delta T_0)/t_2$ to $\eta_0/2$, a value of the latter is obtained which is corrected for the presence of the non-hyaluronic acid materials that influence the viscosity. The application of this correction is illustrated in Fig. 2 which presents the results of experiments with a brain extract which had been shown to contain neither hyaluronidase nor an inhibitor of this enzyme. The absence of inhibitor was demonstrated by the inability of 0.075 M phosphate to decrease the value of *R*. Haas (7) had demonstrated that 0.1 M phosphate can inactivate the inhibitor, and under the present conditions 0.075 M also proved effective. The difference in the value of η between lines *a* and *b*, Fig. 2, represents the magnitude of the correction for the presence of the brain extract which increases the viscosity.

A possibility of error exists either if the enzyme solution can hydrolyze a viscous constituent in the tissue extract, or if the extract contains an appreciable hyaluronic acid concentration. In either event the ΔT value would decrease faster than otherwise. Experiments were performed on liver and kidney extracts in which the usual experimental conditions were employed with the exception that the tissue extracts were substituted for substrate. In neither instance was there any fall in outflow time over an 8 minute period, the duration of most of the viscosity experiments.

Haas (7) has shown that phosphate inhibits the reaction between hyaluronidase and the serum inhibitor. Therefore the difference in *R* values with and without phosphate (correcting for a small inhibitor effect that phosphate has on the enzyme) gives a measure of inhibitor concentration, and this can be used as another method of determining the serum inhibitor in tissues.

In the present work with tissue extracts, control experiments were employed in which the viscosity of a mixture of the tissue extract, hyaluronic acid, and buffer was measured over the experimental period. Whenever there was a question of hyaluronidase inhibitor activity in the tissue extract, phosphate was employed in a parallel experiment to suppress the effect of inhibitor, should any of the latter be present.

Results

Tissues and Body Fluids—No tissue was found that had a detectable amount of inhibitor by the method employed. The tissues listed in Table III were analyzed; each analysis was performed on the tissue from a different rabbit. Haas (7) had examined several hog organs for the presence of inhibitor and he also could detect none. His data on thymus tissue were inconclusive due to the viscous nature of the extract.

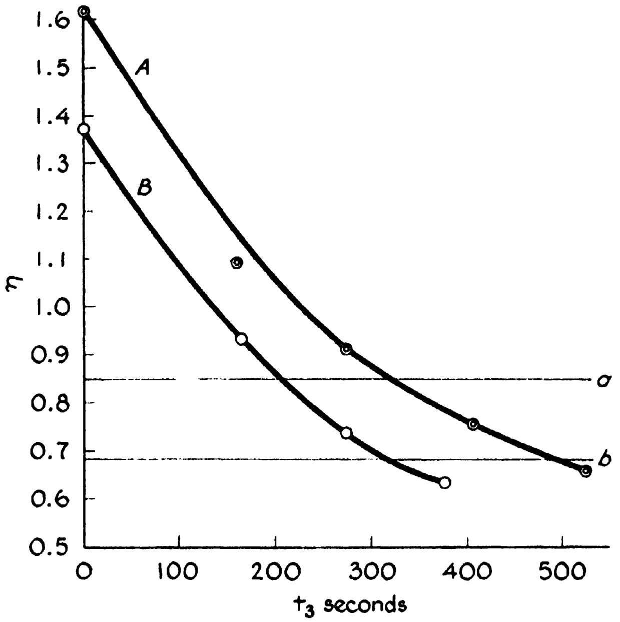

FIG. 2. "Relative viscosity" curves of reaction mixtures with and without added rabbit brain extract. Without brain extract, Curve B, t_2 = 43 seconds, $b = \eta_0/2$, R_0 = 320 seconds. With 1 ml. of brain extract, Curve A, t_2 = 43 seconds, $a = (\eta_0/2) + (0.7\Delta T_0/t_2)$, R_0 = 320 seconds.

In the control experiments employed for the calculation of ΔT, that also serve to detect the presence of hyaluronidase as previously mentioned, none of the enzyme was found in any of the tissues tested, including undescended testes. The hyaluronidase in 1 mg. of descended testes would be readily detected.

The question arises whether the serum present in the tissues would exert an appreciable inhibition. Since it did not, it is conceivable that the tissue effected a destruction of the serum inhibitor present. This, however, appears unlikely since incubation at room temperature for approximately 1 hour of macerated liver, spleen, lung, or bone marrow with added serum in the proportion of 1 part of tissue to 2 of serum did not alter the inhibitor concentration of the serum. Therefore, it would appear that the amount of serum in the tissue samples employed was not great enough to exert a demonstrable inhibitory effect by the method used.

TABLE III

Rabbit Tissues in Which Neither Hyaluronidase Nor Hyaluronidase Inhibitor Could Be Detected

Tissue	No. of analyses	Tissue	No. of analyses	Tissue	No. of analyses
Lymph nodes	2	Kidney	3	Mammary gland (pregnant rabbit)	1
Bone marrow	3	Stomach	3		
White blood cells (buffy coat)	2	Small intestine	3		
		Colon	2	Uterus and tubes	1
Liver	3	Appendix	1	Ovaries	1
Red blood cells	1	Pancreas	2	Undescended testes	1
Thymus	3	Striated muscle	2		
Spleen	2	Brain	2	Adrenals	2
Gallbladder	2	Lung	3	Pituitaries	1
Cartilage	1			Thyroid	1
Perirenal fat	1			Eye glands	1
Skin	1			Salivary glands	2

TABLE IV

Inhibition of Hyaluronidase by Body Fluids

Fluid	No. of specimens tested	Volume used	Observed per cent inhibition	Calculated per cent inhibition per 0.05 ml.
		ml.		
Rabbit serum	8	0.10	44	22.0
" bile (gallbladder)	3	0.02	39	97.5
" urine	2	1.0	38	1.9
" amniotic fluid	1	1.0	0	0
Human saliva	2	1.0	0	0
" spinal fluid	2	1.0	0	0
" milk	2	1.0	0	0
Hog synovial fluid	1	0.25 gm.	0	0

Serum, gallbladder bile, and urine all inhibited hyaluronidase (Table IV); the other body fluids were without effect. It had been shown previously

(12) that bullous fluid from patients with pemphigus contained inhibitor; samples of human ascitic fluid also were found in the present study to contain it. Bile and urine inhibitors are not destroyed by heating at 60° for 20 minutes, and their inhibition is not decreased in 0.075 M phosphate. Both the heat and phosphate inactivate the serum inhibitor. Synovial fluid from the hog was tested, but the high hyaluronic acid concentration in this material complicates the analysis. Nevertheless, the results indicated that no inhibitory activity was present.

Inhibition of Hyaluronidase by Hemoglobin Derivatives, Bile Salts, and Sterol Hormone Conjugates—Hemoglobin, its derivatives, the bile salts, and estradiol phthalate were first dissolved in a small volume of 0.1 N NaOH, neutralized with dilute HCl to approximately pH 8.0 and then diluted with several volumes of 0.1 M veronal buffer of pH 7.4. Bilirubin, in the concentrations used, precipitated under these conditions and hence was used in an unbuffered solution of pH 8.0. The sterol hormone conjugates, with the exception of estradiol phthalate, were dissolved directly in the veronal buffer. The effect of the compounds on hyaluronidase is presented in Table V, and the relationship between concentration and hyaluronidase inhibitions for certain representative compounds is given in Fig. 3.

In addition to the sterol conjugates listed in Table V, several more difficultly soluble sterols were tested both in aqueous solution and after homogenization in a Potter-Elvehjem homogenizer for 10 minutes with serum inactivated by heating at 60° for 20 minutes. The compounds were testosterone propionate (Ciba), anhydrohydroxyprogesterone (Ciba), Kendall's Compound E (Kendall), ethinylestradiol (Ciba), and desoxycorticosterone acetate (Schering). These were without effect.

Sodium glucuronate (0.5 mg. per ml.), sodium phthalate (0.08 mg. per ml), and sodium sulfate (0.75 mg. per ml.) both alone and in the presence of serum showed no effect on hyaluronidase. Sodium phthalate, in the concentration employed, inhibited the serum inhibitor slightly.

The hyaluronidase-inhibitory activity of hemin, biliverdin, protoporphyrin, sodium glycocholate, and sodium androsterone sulfate were also studied in the presence of 0.075 M phosphate, and no change in inhibitory activity was observed.

Inhibition of Serum Inhibitor—The compounds listed in Table V were also tested in the presence of normal human serum. The compound was first mixed with the serum, diluted to the appropriate volume, and then the enzyme was added. Those compounds which were without inhibiting effect on hyaluronidase when tested in the absence of serum were also inactive in its presence. In the case of the hyaluronidase-inhibiting hemoglobin derivatives and bile salts, and to a lesser extent some of the sterol hormone conjugates, it was found that the inhibition of the serum inhibitor and compound when mixed together was far lower than the sum of their separate inhibitions.

TABLE V

Inhibition of Hyaluronidase by Hemoglobin Derivatives, Bile Salts, and Sterol Hormone Conjugates

Compound	Concentration	Per cent inhibition observed	Calculated per cent inhibition per 0.1 mg.
	mg. per ml.		
Hemin* (Armour)	0.15	66	44.0
Protoporphyrin*†	0.24	34	14.1
Coproporphyrin* III†	0.33	20	6.1
Bilirubin*†	0.38	73	19.2
Biliverdin*†	0.28	35	12.5
Stercobilin*†	0.80	0	0
Hemoglobin (Armour)	7.5	0	0
Bilirubin protein compound from Fraction V-2 (Squibb)	8.4	0	0
Sodium androsterone sulfate* (Ciba)	0.85	44	5.2
Sodium dehydroisoandrosterone sulfate* (Ciba)	0.50	26	5.2
Sodium pregnanediol glucuronidate*‡	0.70	34	4.9
α-Estradiol-17-phthalate*§	0.20	21	10.1
Estrone sulfate* (Ayerst, McKenna and Harrison, Ltd.)	0.65	0	0
Sodium glycocholate (Ames)	5.0	57	1.1
Sodium taurocholate†	20.0	51	0.26
Sodium desoxycholate (Ames)	0.70	58	8.3
Sodium cholate (Ames)	5.0	35	0.7

* Crystalline.

† Prepared in the laboratories of Dr. C. J. Watson, Department of Medicine, University of Minnesota.

‡ Prepared by Dr. Saul Cohen, Department of Physiological Chemistry, University of Minnesota.

§ Prepared by Dr. W. H. Pearlman, Jefferson Medical College, Philadelphia.

In Table VI the inhibition of the serum inhibitor by the compounds found to have this property is demonstrated. Thus, hemin, protoporphyrin, bilirubin, and the bile salts in certain concentrations form serum solutions whose hyaluronidase inhibitions are less than that of the serum alone. Stercobilin, which did not inhibit hyaluronidase, was found to inhibit the serum inhibitor. On the other hand, the serum inhibitor was not inhibited at any of the concentrations of hemoglobin (0.05 to 25 mg.

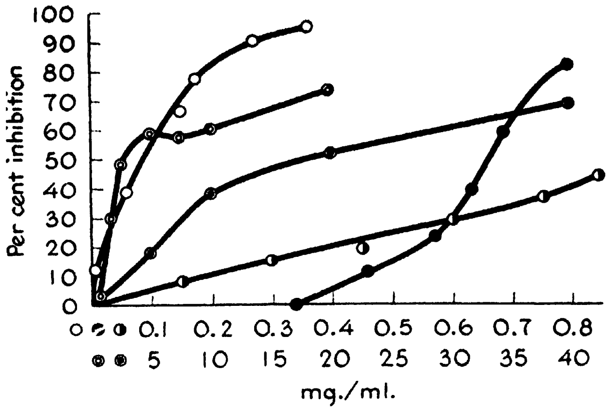

Fig. 3. Effect of concentration of certain compounds on hyaluronidase inhibition. Hemin (○), sodium androsterone sulfate (◑), sodium desoxycholate (●), sodium glycocholate (◎), sodium taurocholate (◉). Solubility limitations did not permit measurements with sodium androsterone sulfate at higher concentrations.

Table VI

Inhibition of Hyaluronidase by Certain Compounds in Presence and Absence of Human Serum

Compound	Concentration	Per cent inhibition observed			Per cent decrease of inhibition from additive value of compound + serum	Per cent decrease of inhibition of compound in presence of 0.1 ml. heated serum
		Compound	Serum* (0.075 ml.)	Compound + serum		
	mg. per ml.					
Hemin	0.18	77	38	27	77	83
"	0.36	94	38	45	66	
Protoporphyrin	0.24	34	64	48	51	71
Bilirubin	0.08	13	66	62	22	
"	0.38	73	66	69	50	77
Biliverdin	0.28	35	27	36	42	
Stercobilin	0.80	0	29	16	45	
Sodium androsterone sulfate	0.60	29	33	53	14	
" " "	0.85	44				29
" dehydroisoandrosterone sulfate	0.50	26	52	59	24	
" pregnanediol glucuronidate	0.70	34	45	64	19	
α-Estradiol-17-phthalate	0.20	21	57	61	21	
Sodium glycocholate	1.5	27	44	27	62	
" "	5.0	57	31	46	48	68
" taurocholate	0.5	0	40	35	12	
" "	40.0	68	40	65	40	
" desoxycholate	0.7	58	43	26	75	
" cholate	5.0	35	35	27	61	

* Different samples of serum were used for the various experiments.

per ml.) or sodium androsterone sulfate (0.15 to 0.85 mg. per ml.) used. Other sterol hormones, and coproporphyrin and biliverdin were not tested to evaluate their effects on the serum inhibitor due to the scarcity of the compounds.

Decrease in Hyaluronidase-Inhibitory Activity of Various Compounds in Presence of Serum—The loss of hyaluronidase-inhibitory activity of the compounds studied due to serum is demonstrated in Table VI in two ways.

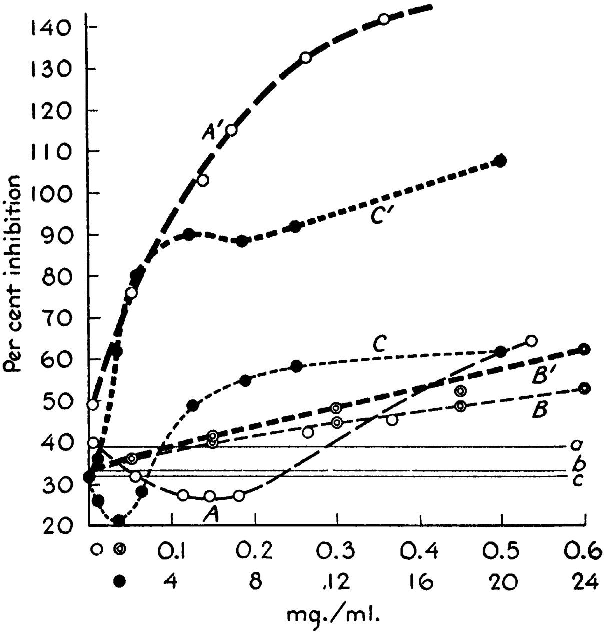

FIG. 4. Effect of concentration of certain compounds in the presence of human serum on the hyaluronidase-inhibitory activity. Hemin (Curves A and A'), sodium androsterone sulfate (Curves B and B'), sodium glycocholate (Curves C and C'). Inhibition by serum alone, lines *a*, *b*, and *c*. Experimental values, Curves A, B, and C; calculated values, Curves A', B', and C'. Curves A' and C' exceed 100 per cent, since they represent inhibition as per cent of the enzyme in a reaction mixture theoretically capable of being inhibited.

First, the addition of unheated serum to certain compounds resulted in solutions which had less hyaluronidase-inhibitory activity than the compound alone in the same concentration, *e.g.* hemin, desoxycholate, and cholate. Second, the addition of serum, in which the inhibitor had been destroyed by heating at 60° for 20 minutes, to a number of the compounds also resulted in a solution with less inhibitory activity than that of the compound alone. It will be noted that certain of the sterol hormone conjugates retain considerably more hyaluronidase-inhibitory activity than the other compounds after dissolving them in serum.

Effect of Concentration of Inhibitor Compounds on Hyaluronidase Activity in Presence of Serum—The effect of concentration of three representative compounds dissolved in serum is shown in Fig. 4. At all of the concentrations of sodium androsterone sulfate, an increased inhibitory effect over that of serum alone was observed, whereas with hemin and sodium glycocholate there was first a decrease, and then an increase of the inhibition. Sodium androsterone sulfate and sodium pregnanediol glucuronidate, when added to serum, come nearest to giving mixtures which have inhibitory activities equal to those of the sums of the inhibitory activities of the separate constituents.

DISCUSSION

Three groups of compounds, sterol hormone conjugates, hemoglobin derivatives, and bile salts, have been shown to inhibit hyaluronidase. Consideration of the concentrations of these compounds in circulating blood and their hyaluronidase-inhibiting effects at these concentrations would lead to the conclusion that, individually, these compounds contribute little to the total inhibitor content of the blood serum, but all together they might exert an effect. There is also the possibility that they may play an inhibitory rôle in local tissue sites, *e.g.* the cells of the reticuloendothelial system where the products of hemoglobin breakdown and hyaluronidase from phaged bacteria may coexist, in sites of tissue destruction where high concentrations of porphyrin have been observed, etc. These compounds differ from the labile serum inhibitor in their greater heat stability, and in the fact that their inhibitory activity is not decreased in the presence of phosphate.

Certain effects of structure of the compounds studied on their hyaluronidase-inhibiting properties may be deduced from the data obtained. The fact that hemoglobin produced no inhibition, whereas hemin and protoporphyrin did inhibit, suggests that the large globin moiety in the former may have prevented, by steric hindrance, the combination of the inhibiting prosthetic group with the enzyme. Competitive inhibition might be expected from certain acid compounds, since the substrate of the enzyme is itself acidic. However, when the number of carboxyl groups in the protoporphyrin molecule is doubled by replacing the two vinyl groups with propionic acid radicals, thus converting the compound to coproporphyrin III, the inhibiting effect is reduced. The constitutional difference between hemin and protoporphyrin is the presence of iron in the former but not in the latter; this difference is reflected in the markedly greater inhibitory effect of the hemin.

The inhibitions produced by bilirubin and biliverdin are not very different, but the replacement of the vinyl groups in the latter by ethyl groups and the saturation of double bonds in the terminal pyrrole rings to form stercobilin are accompanied by the loss of all inhibitory activity. Removal of a hydroxyl group from the cholic acid nucleus to form the desoxycholic acid results in an increase of the inhibitory effect. Alterations in the chain attached to the 17-carbon atom, as in glycocholate and taurocholate, have little effect. Sodium androsterone sulfate has inhibiting properties, but replacement of the saturated Ring I with a benzene ring to form estrone sulfate results in the loss of this inhibition. However, the reduction of estrone to estradiol effects a recovery of the inhibiting property. The introduction of a double bond in Ring II of androsterone to form dehydroisoandrosterone has no effect on inhibiting capacity.

Stercobilin, bilirubin, protoporphyrin, hemin, and the bile salts have been found to inhibit the serum hyaluronidase inhibitor; however, their serum concentrations *in vivo* would appear to be too low to be significant. Nevertheless, the combined effects of a group of these compounds might be of physiological importance, particularly in conditions accompanied by elevated levels of these substances, and *in vivo* effects might be appreciable in local tissue sites. Since these compounds with the exception of stercobilin can both inhibit hyaluronidase and the serum inhibitor, their resultant effect would depend on the particular combination of compounds present and their respective concentrations.

It is interesting to note that stercobilin which has certain of the properties of Haas' (18) "proinvasin I," *i.e.* it inhibits the serum inhibitor without inhibiting hyaluronidase, should be the product of bacterial metabolism of a bile pigment. Thus a potential inhibitor of bacterial hyaluronidase is converted in the intestines to a compound that aids their invasiveness.

The clinical observation (19) of the improvement of patients with rheumatoid arthritis by an accompanying jaundice or pregnancy is of interest in connection with the present work. In jaundice there exists increased concentrations of compounds which we have found to affect hyaluronidase, and which might therefore have some influence on the hyaluronic acid of the affected joints. Of course this presupposes that hyaluronidase is involved in the pathological development, and that remains to be established. Too little is known concerning the blood levels of hormones in pregnancy to draw any conclusion as to their rôle as hyaluronidase inhibitors in this state. Hakanson and Glick (20) in fact found no change in the inhibitor level of the blood serum during normal pregnancy in the human.

The lack of demonstrable hyaluronidase inhibitor in any of the tissues studied indicates one of the following possibilities: that there is no appreciable accumulation of the inhibitor in the tissues, that it is not extracted from them by the technique used, or that the inhibitor is formed in the blood stream itself.

SUMMARY

The hyaluronidase-inhibitory activity of certain sterol hormone conjugates, bile salts, and hemoglobin derivatives has been demonstrated.

On the basis of heat stability and retention of inhibitory activity in the presence of phosphate these inhibitor compounds may be differentiated from the serum inhibitor.

In the presence of serum these compounds show a decrease in their inhibitory activity, this being least for certain of the sterol hormone conjugates. Certain of the hemoglobin derivatives and all of the bile salts inhibit the serum hyaluronidase inhibitor.

Sodium androsterone sulfate differs from the hemoglobin derivatives and bile salts in that it does not inhibit the serum hyaluronidase inhibitor and is itself inhibited by serum to a smaller degree.

Of the compounds tested, stercobilin has the unique property of inhibiting the serum inhibitor, and not inhibiting hyaluronidase itself.

A viscosimetric method for the quantitative analysis of the serum inhibitor in the presence of materials that increase viscosity such as tissue extracts has been described.

No tissue has been found which contains a significant amount of hyaluronidase inhibitor.

In addition to serum, the only body fluids investigated in this study that were found to inhibit hyaluronidase are bile and urine. The inhibitors present in the latter are distinct from that in the former as indicated by their relative thermostability and independence of phosphate.

Relationships between the structure of the compounds investigated and their inhibiting effects have been discussed.

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EFFECT OF TRACE IMPURITIES IN ADENOSINE TRIPHOSPHATE*

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Adenosine triphosphate (ATP) is a necessary component of the enzyme system that oxidizes pyruvate and oxalacetate in homogenates of certain animal tissues (1, 2). Muscle adenylic acid is also effective, since it is readily converted to ATP. Attempts to confirm or apply the results of these studies may be seriously hampered by the use of commercial samples of ATP if these preparations contain trace impurities, which are not revealed by determinations of ribose, nitrogen, total phosphate, inorganic phosphate, and labile phosphate. One such example has already come to our attention. In the enzyme system that we employ, the amount of tissue in the reaction mixture is very small, and since, as will be shown, the per cent inhibition increases as the amount of tissue decreases, this enzyme system is a sensitive indicator of trace impurities in ATP preparations.

Since ATP is an important component of the reaction mixture in many enzyme systems that are of current interest, it seems desirable to call attention to the fact that various commercial samples contain trace inhibitors, to show how these inhibitors may be easily revealed, and to report that, in the case of the preparations examined, the impurities were eliminated by purification following standard procedures (3). It is thus likely that improved preparations can be made available commercially.

EXPERIMENTAL

Test System—The enzyme system employed is essentially that of Potter, Pardee, and Lyle (2), modified in order to make it more widely available for the present type of test. To this end, fumarate may be substituted for oxalacetate and cytochrome *c* may be omitted. The amounts of ATP and homogenate are varied for the purposes of the assay. The reaction components are as follows: water to make a final volume of 3.0 ml., 0.4 ml. of 0.5 M KCl, 0.1 ml. of 0.1 M $MgCl_2$, 0.5 ml. of 0.1 M K phosphate of pH 7.2, 0.4 ml. of 0.05 M K pyruvate, 0.4 ml. of 0.04 M K fumarate, 0.3 to 0.5 ml. of 0.01 M K ATP, and 0.1 to 0.4 ml. of 10 per cent kidney homogenate

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in isotonic KCl plus bicarbonate (1). All components are kept cold until the flasks are placed in the 38° bath. Oxygen uptake is measured for about six successive 10 minute intervals in Warburg flasks with no side arms.

Each reaction mixture is set up in duplicate and the results are the average of the two successive 10 minute periods that represent the maximum rate. Each preparation of ATP is tested at two levels of tissue, 0.2 and 0.3 ml. of 10 per cent homogenate, with 0.5 ml. of 0.01 M ATP, and at two levels of ATP, 0.3 and 0.5 ml. of 0.01 M, with 0.3 ml. of homogenate. The minimum test thus requires six flasks per sample.

Preparations—The pyruvate is prepared by diluting 1 N acid, neutralizing it with K_2CO_3, and removing the CO_2 by aeration. The 1 N pyruvic acid is kept in the cold and is prepared by double distillation of Eastman pyruvic acid at 2 to 4 mm. of Hg, with immediate dilution of the product. The fumaric acid was obtained from Eimer and Amend. Two preparations of ATP were prepared in this laboratory by G. A. LePage and by W. W. Ackermann, according to methods earlier described (3). They are designated as Preparations GAL and WWA in Figs. 1 and 2. Two commercial samples of ATP are designated Preparations C-1 and C-2. These samples were partially but not completely purified by precipitating $BaSO_4$ in a solution of the preparation as described earlier (3). Repetition of this procedure was ineffective. The two preparations were then put through the entire purification procedure (3), yielding samples designated as Preparations P-1 and P-2.

The kidney tissue was obtained from young adult male rats obtained from the Holtzman Rat Company, and maintained on a stock diet of mixed grains.

Results

The results are shown in Figs. 1 and 2. While the data include preparations that are considered to be primary standards, an impure preparation may be recognized without the use of a primary standard.

In Fig. 1 the rate of oxidation is plotted against the concentration of ATP. In the case of Preparations GAL, WWA, P-1, and P-2, which are relatively pure, an increase in ATP concentration produced no change in the rate after the maximum rate had been reached. In the case of Preparations C-1 and C-2, which were tested as received, the rate of oxidation *decreased* when the amount of ATP was *increased*, showing that impurities were present. These results show that the inhibition is due to the ATP preparation and not to any other reaction component.

In Fig. 2 the rate of oxidation is plotted against the concentration of tissue. In the ideal case a straight line through the origin should be obtained. When Preparations GAL, WWA, P-1, and P-2 were used, the results were essentially the same and yielded a line that passed through a point slightly to the right of the origin. When Preparations C-1 and C-2 were used, a marked shift to the right occurred, yielding lines that were parallel to the control data. Partially purified samples of Preparations 1 and 2 gave lines that fell between the control data and the data obtained with untreated samples. These data are almost identical with similar data obtained by Ackermann and Potter[1] using the succinoxidase system

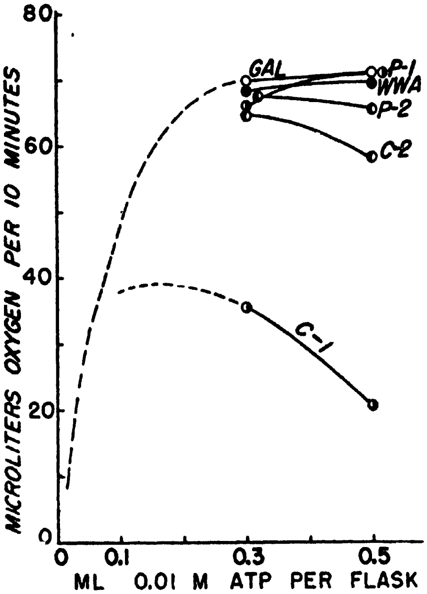

FIG. 1

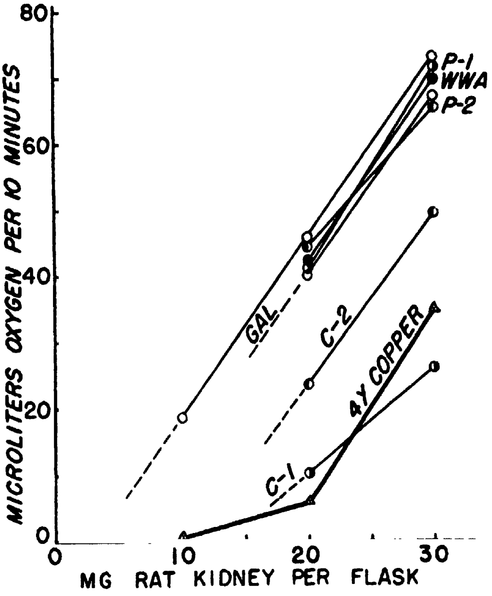

FIG. 2

FIG. 1. Effect of ATP concentration on the rate of oxidation of pyruvate plus fumarate. See the text for reaction components. Various samples of ATP were used: Preparations C-1 and C-2 were commercial samples before purification; Preparations P-1 and P-2 were the same samples after purification; Preparations GAL and WWA were made in this laboratory. Preparation GAL was tested in parallel with each of the other samples, but the variation between homogenates was so slight that data from only one experiment are reported.

FIG. 2. Effect of enzyme concentration upon the inhibition produced by 0.5 ml. of 0.01 M ATP. See the text for reaction components of oxidative system. The various curves were obtained with the same ATP preparations used to obtain the data in Fig. 1. Each sample was compared with a primary standard (Preparation GAL). Only two experiments with Preparation GAL are shown, since the other data fell within the range represented by the experiments reported. The results obtained in the presence of 4 γ of copper added as copper sulfate to a system containing 0.5 ml. of ATP (Preparation GAL) are included as another example of inhibition in which the per cent inhibition depends on the tissue concentration (*cf.* (4)).

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[1] Ackermann, W. W., and Potter, V. R., unpublished work.

in the presence of 1 or 2 γ of copper per flask. Experiments were carried out with the present reaction system with various levels of copper, mercury, cadmium, iron, and nickel. At final concentrations of 2×10^{-5} M, mercuric and cupric ions gave about the same degree of inhibition, cadmium was much more toxic, and ferric and nickelous ions were not toxic. Fig. 2 shows the effect of adding 4 γ of copper per flask (2×10^{-5} M) at various tissue concentrations. The data correspond to what might be expected from an inhibitor that is essentially undissociated from the enzyme,[1] in which case the inhibitor has the effect of eliminating an absolute amount of enzyme that is independent of the enzyme concentration. The per cent inhibition accordingly decreases with increasing enzyme concentration. Whether the inhibition produced by the impure samples of ATP is due to heavy metals cannot be concluded from these data. Calcium ions have also been shown to inhibit the enzyme system (4). The slight displacement of the control curves from the origin is believed to be due to impurities in the system other than in the ATP, since increasing the concentration of the control ATP preparations did not increase the inhibition (Fig. 1).

The results show that the impurities can be removed by the purification procedure previously employed (3). We have been advised by Dr. J. A. Bain of the University of Illinois College of Medicine that when this test was applied to five samples of commercial ATP only one proved satisfactory. We have found one sample of commercial muscle adenylic acid to be satisfactory.

This test does not determine whether a preparation of ATP contains adenosine monophosphate or adenosine diphosphate but this problem is adequately handled by chemical analyses. In this connection the report (5) that ATP spontaneously breaks down to adenosine monophosphate and inorganic pyrophosphate does not appear to apply to all samples. In the above report the conditions of storage were not specified. Samples of ATP (Ba salt) made in this laboratory are dried *in vacuo* and held in a desiccator over $CaCl_2$. The salt is placed in several small containers (3 to 4 gm.) which are used up one at a time. In the course of ATP analyses reported earlier (1) the fractionation was checked with reference to the behavior of inorganic pyrophosphate. At the levels at which ATP was used any inorganic pyrophosphate present in the ATP would have appeared in the barium-soluble fraction instead of in the barium-insoluble fraction in which the ATP was found. No evidence of inorganic pyrophosphate was found in a preparation that was 4 years old.

SUMMARY

1. Trace impurities in adenosine triphosphate (ATP) obtained from commercial sources were shown to produce marked inhibition of the rate

of oxygen uptake by rat kidney homogenates in the presence of pyruvate and fumarate.

2. When the concentration of the impure samples was increased, the oxygen uptake decreased, and when the rate of oxygen uptake was plotted against tissue concentration, the result was a line parallel to but lower than the line obtained with control data.

3. The impure preparations were purified by standard methods to yield satisfactory products.

The authors wish to acknowledge the technical assistance of Gloria G. Lyle in carrying out measurements of oxygen uptake.

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A COLORIMETRIC METHOD FOR THE ESTIMATION OF ACETOACETIC ACID IN THE BLOOD*

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It was observed that acetoacetic acid when coupled with 4-nitrobenzenediazonium salt in a buffered carbonate solution yielded a strong green color upon further alkalization. This reaction was given by relatively few of the biochemical compounds that have been tested, and the procedure has been developed so that, as applied to blood filtrates, it is believed to afford a satisfactory measure of acetoacetic acid content.

Nature of Reaction

Azo compounds of ketones have long been known and the derivative of 4-nitrobenzenediazonium salt with acetoacetic acid has been described (1). Isomeric forms which include the hydrazones, as well as multiple substitutions to yield formazyl compounds, have also been described (2). The Arnold test for acetoacetic acid in the urine is based upon a diazo reaction (3).

The reaction products in our procedure have not been identified, but the following evidence indicates that the hydrazone is not involved: The hydrazone of acetoacetic acid has a rose color with an entirely different absorption spectrum (4); the hydrazone of acetoacetic acid is reported to have a color intensity only 6 per cent of that of pyruvic acid (4), while in our procedure the green color is approximately 10 times as strong as equimolecular quantities of pyruvic acid; the hydrazone reaction is given by α-keto acids, while in this method, with the exception of pyruvic acid, those tested have given negative responses.

The absorption spectra of the azo compounds of acetoacetic and pyruvic acids in a butanol-benzene solvent are shown in Fig. 1. Two peaks of absorption suggested the possibility of two components, and this was further indicated by passing the green butanol-benzene extract from acetoacetic acid through an activated alumina column 15 cm. in length. Upon alkalization of the filtrate a blue component was first obtained, followed by a yellow component which appeared upon washing the column with butanol. A large part of the absorption occurring below 470 mμ, and extending into the ultraviolet, is contributed by the reagent blank extracts.

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The blue component, with maximum absorption at wave-lengths of 640 to 650 mμ, forms the basis of our measurements in the test. Preliminary experiments indicate that this color characteristic is a function of the nitro group in diazotized 4-nitroaniline. Acetoacetic acid reacted with the diazonium salt of sulfanilic acid to give a pale rose solution, not extractable with butanol; with 4-chloroaniline a deep rose color, completely extractable by the butanol-benzene solvent, was obtained.

Method

Whole blood or plasma, oxalated or heparinized, is deproteinized by the zinc procedure of Weichselbaum and Somogyi (5). Since small volumes of filtrate are desirable, 10 per cent $ZnSO_4 \cdot 10H_2O$ and approximately 0.4 N $Ba(OH)_2$ are used. To 5 cc. of blood or plasma are added 10 cc. of water, then the amount of $Ba(OH)_2$[1] as shown to be required by titration against the zinc solution (5), and, after mixing, 5 cc. of the $ZnSO_4$ solution. The tube is corked and vigorously shaken. Before filtering, the tubes are centrifuged to increase the quantity of filtrate.

Three tubes are used in the test. In one tube are placed 5 cc. of the filtrate plus 0.5 cc. of 5 N H_2SO_4; this tube is immersed in boiling water for 5 minutes, then cooled in ice water, and neutralized with 0.5 cc. of 5 N NaOH. 5 cc. of filtrate are placed in the second tube, and 5 cc. of water in the third tube for the reagent blank. When high concentrations of acetoacetic acid occur, smaller aliquots of the filtrates are taken and made up to 5 cc.

The tubes are placed in ice, and 4 cc. of the buffer mixture added and mixed by agitation; 2 cc. of the diazo reagent are added, and the tubes agitated and allowed to remain in the ice bath for exactly 10 minutes. 1 cc. of 5 N NaOH is now added, and the tubes are agitated and replaced in the ice bath for 2 minutes. 5 cc. of butanol-benzene solvent are now added, and the tubes tightly stoppered with corks and shaken.

The tubes are centrifuged for 1 to 2 minutes, and, by means of a pipette with a rubber bulb, 3 cc. of the solvent are pipetted into small colorimeter tubes containing 0.5 cc. of 0.05 N NaOH in absolute ethanol. The colorimeter tubes are stoppered and read in a spectrophotometer at a wavelength of 640 to 650 mμ, or in a colorimeter equipped with an appropiate filter.

If the available amount of blood filtrate is not sufficient, all volumes can be reduced proportionately.

Standard curves are obtained by using 5 cc. of a solution containing 0.25 to 4 γ of acetoacetic acid per cc.; these dilutions are made up in a

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[1] The $Ba(OH)_2$ must be frequently titrated against the $ZnSO_4$, as $BaCO_3$ precipitates on standing.

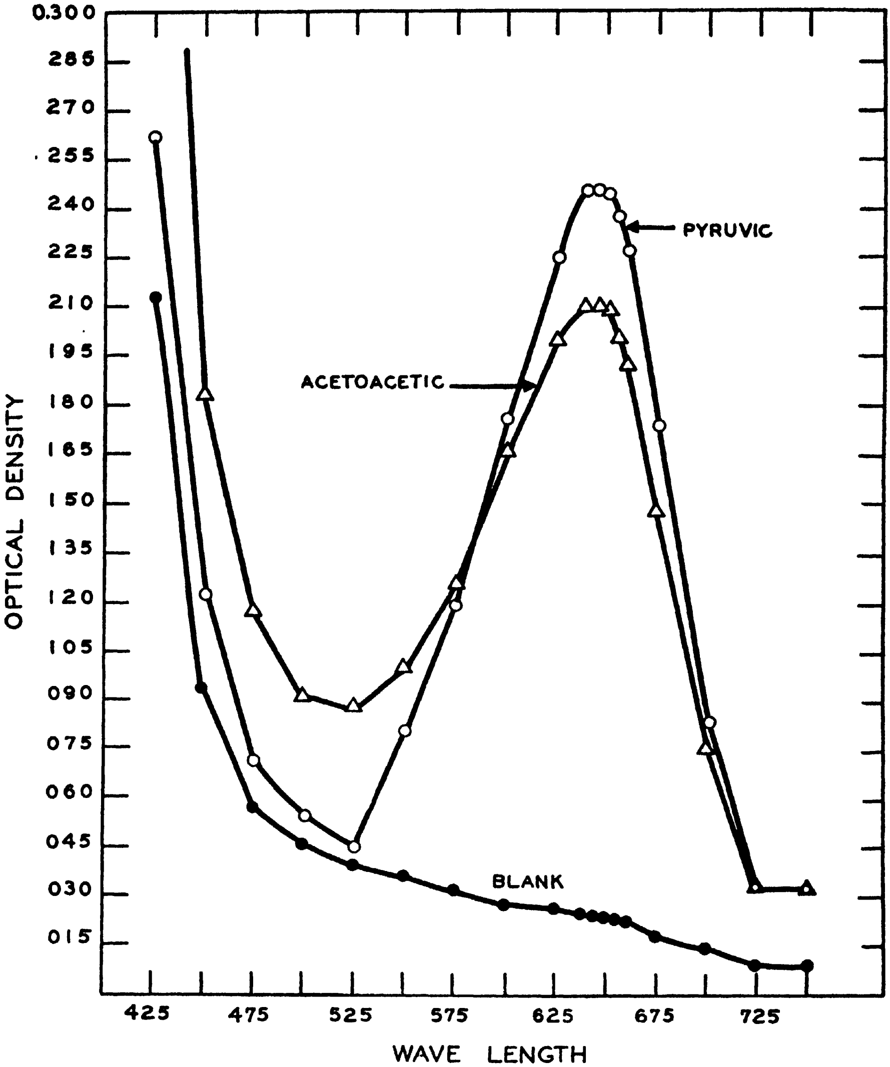

FIG. 1. Absorption spectra upon butanol-benzene extracts from tests carried out upon solutions containing 1 γ per cc. of acetoacetic acid and 10 γ per cc. of pyruvic acid. Determinations carried out with a Beckman spectrophotometer with cells 1 cm. in length.

filtrate obtained by carrying out the zinc precipitation in water alone. The readings of the reagent blank must be deducted from all readings, unless the comparisons are made against the reagent blank.

The extraction with the butanol-benzene solvent is not complete, so that the calibration curve is not linear. However, the use of benzene does away with many extraneous colors, and it was considered preferable to obtain a low reagent blank at the expense of incomplete extraction. For unknown reasons, repeated extraction with this mixture does not take up the remaining green color in the aqueous phase.

The values for the filtrates are obtained from the standard curves; after multiplication by the dilution factor, the reading of the boiled filtrate is subtracted from that of the unboiled, to obtain the amount of acetoacetic acid per cc. of blood.

Reagents Required—Distilled water[2] that is free from copper should be employed. Special care is also required that glassware be clean and free from copper; overnight soaking in cleaning fluid, followed by rinsing in tap water and a final rinse in distilled water, has been found adequate. The chemicals should all be of reagent grade.

Buffer solution. It has been found preferable to make stock solutions of the individual ingredients and mix them in proper proportion for daily use: mix together 1 part of 1 M oxalic acid and 1 part of 1 M tribasic potassium phosphate; add 2 parts of 50 per cent potassium carbonate. The mixture is cooled in ice and 4 cc. added to 5 cc. of the cooled blood filtrate.

Diazo reagent. This is prepared from a stock solution of 0.1 per cent 4-nitroaniline in 0.1 N sulfuric acid; it is necessary to heat to boiling to bring this chemical into solution. The reagent is prepared freshly a few minutes before use by cooling 10 cc. of this solution in ice and then mixing with 1 cc. of 4 per cent sodium nitrite.

Other solutions required are 10 per cent $ZnSO_4 \cdot 10H_2O$, 0.4 N $Ba(OH)_2$, 5 N H_2SO_4, 5 N NaOH, a mixture of equal parts of *n*-butanol and thiophene-free benzene (C_6H_6), and an alcoholic NaOH solution made by adding 0.5 cc. of 5 N NaOH to 50 cc. of absolute ethanol (this is conveniently kept in a large cork- or glass-stoppered test-tube so that it can be centrifuged if any turbidity is present).

A 2 per cent solution of acetoacetic acid for use as a standard has been prepared (as the sodium salt) every few weeks and kept in the refrigerator: 1.3 cc. of ethyl acetoacetate (redistilled) are added to 51 cc. of 0.2 N NaOH and allowed to stand in the refrigerator 2 days before using. 0.5 cc. of this solution diluted to 1000 cc. yields a solution containing 10 γ per cc. Ethyl acetoacetate gives a different calibration curve and cannot be used as a standard.

Specificity of Method

The following compounds[3] react to give a green color with an absorption peak at 640 to 650 mμ; their approximate relative intensities (on a molar basis) are as follows: (1) acetoacetic acid 100, (2) ethyl acetoacetate > 100, (3) oxalacetic acid 100, (4) α,γ-diketovaleric acid 100, (5) acetylacetone 115, (6) pyruvic acid 10, (7) tyrosine 20, (8) guanine 1.5, (9) thymine 1.5.

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[2] Water obtained by the use of a Barnstead bantam demineralizer has been found satisfactory.

[3] Several of the keto acids were supplied by Dr. A. Kornberg, Dr. A. Meister, and Dr. H. W. Bond. The oxalosuccinic acid was originally prepared by Dr. S. Ochoa.

Among many compounds tested (in amounts from 100 to 1000 γ), those that did not give a significant reaction included acetone, acetonylacetone, oxalosuccinic acid, α-ketoglutaric acid, levulinic acid, α-ketoisocaproic acid, dehydroacetic acid, β-hydroxybutyric acid, malonic, maleic, and malic acids.

With the exceptions (6) to (9) shown above, the principal reacting group appears to require the linkage $CC(=O)CH_2C(=O)$—. Several α-alkyl monosubstitution products of acetoacetic acid also reacted positively. The negative response of oxalosuccinic acid may be due to its instability.

Of the β-keto acids which respond, only acetoacetic acid is believed to be in the blood filtrates in measurable amounts. Should it be desired to employ the method to demonstrate acetylacetone or α,γ-diketovaleric acids, these compounds may be differentiated by their rate of destruction on heating in acid solution. While acetoacetic and oxalacetic acids are rapidly destroyed, the rate is very much slower with acetylacetone, and moderately slower with α,γ-diketovaleric acid. It should also be noted that oxalacetic acid is changed to pyruvic acid by this treatment; so that the heated solution gives a color reaction equivalent to approximately 10 per cent of the original.

Since the other compounds known to give the reaction are resistant to brief heating in acid solution, it is believed that the fraction destroyed by this heating affords an accurate measure of acetoacetic acid in blood filtrates.

The evidence so far obtained indicates that the residue of color absorption at 640 to 650 mμ that remains after heating the acidified solution is contributed mainly by pyruvic acid. The other known reacting compounds, in amounts possibly encountered in blood, are retained in the zinc-barium precipitate. When 50 γ of tyrosine, or 100 γ of guanine or thymine, were added per cc. of blood, none could be detected in the filtrate. With 100 γ of tyrosine per cc., approximately 5 per cent appeared in the filtrate.

The special requirements and limitations of the procedure as applied to pyruvic acid are discussed below.

Several amino acids, phenols, and amines react under the conditions of the test to give a rose color. The procedure was originally designed to detect certain aliphatic amines and diamines[4] after preliminary purification, as carried out for histamine (6, 7). These colors fall at another part of the spectrum, and it will be shown below that with a spectrophotometer (or with suitable filters) these colors do not interfere with determinations of acetoacetic acid upon blood filtrates.

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[4] Unpublished data.

The method cannot be applied to tissues or to urines without preliminary purification of the extracts because of interfering colors. Procedures for this purpose have not yet been developed.[5]

TABLE I

Recovery of Acetoacetic Acid (AA) Added to Whole Blood or Plasma

	AA recovered		AA recovered
	γ *per cc.*		γ *per cc.*
Rat whole blood	3.6	Rabbit whole blood	1.05
+ 1 γ AA per cc.	4.7	+ 2 γ AA per cc.	3.15
+ 2 " " " "	6.0	+ 3 " " " "	3.95
+ 3 " " " "	6.8	Plasma	1.05
Whole blood	2.65 (2.7)*	+ 1 AA per cc.	2.15
+ 1 γ AA per cc.	3.6 (3.5)	+ 3 " " " "	3.95
+ 2 " " " "	4.5 (4.6)		
+ 3 " " " "	5.5 (6.0)		
Human whole blood	1.3	Human plasma	1.4
+ 2 γ AA per cc.	3.2	+ 2 γ AA per cc.	3.5
Whole blood	2.6	Plasma	2.6
+ 2 γ AA per cc.	4.8	+ 2 γ AA per cc.	4.8

* The figures in parentheses represent separate determinations on the same filtrates.

Results on Blood Filtrates

In the earlier part of this work a Coleman spectrophotometer (junior model) was used; later a Beckman (model DU) was employed.

The blood samples were immersed in ice upon collection and tests carried out as soon as possible. In Table I is shown the recovery of 1 to 3 γ of acetoacetic acid added to heparinized blood or plasma of several species. Values within 10 per cent of theory were obtained.

In Table II is shown the acetoacetic acid content of whole blood and plasma of the mouse, rat, guinea pig, and man. The animals were young adults of mixed sexes, not fasted except when indicated. Mice and rats were kept upon a commercial laboratory pellet diet, while the guinea pigs were fed oats and cabbage. Human blood samples were obtained from adult male laboratory workers in mid-morning, after breakfast.

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[5] Satisfactory purification of certain tissue extracts has been accomplished by passing the zinc filtrates through a 1 × 5 cm. column of Amberlite IR-120 (Rohm and Haas Company, Philadelphia). The Amberlite requires preliminary washing with 1 N H_2SO_4 followed by several washings with water. Deproteinized filtrates from tissues are obtained by using 1½ to 2 times the amounts of $ZnSO_4$ and $Ba(OH)_2$ that are employed for blood, and water to make a final dilution of 1:10. The tissue is thoroughly ground in a mortar with acid-washed sand and the calculated amounts of $Ba(OH)_2$ and water. The zinc solution is then added with additional stirring. The material is then poured into test-tubes, shaken, briefly centrifuged, and filtered.

TABLE II

Acetoacetic Acid Content of Whole Blood and Plasma in Several Species of Animals

The results are expressed in micrograms per cc. Those for mice were upon pooled samples collected from five to ten mice. The values of whole blood and plasma opposite each other imply the same sample.

Rat		Mouse	
Whole blood	Plasma	Whole blood	Plasma
3.8	4.1	2.45	2.65
4.5	6.3	6.8	7.4
2.7			
2.3		Fasted 25 hrs.	
	5.7		
	8.4	50	62
3.5		141	150
3.5			
3.0		Fasted 48 hrs.	
			290
Fasted 18 hrs.			
42.5			
Fasted 24 hrs.			
66	66		
Man		**Guinea pig**	
0.95	0.97	2.3	2.7
0.55	0.80	1.4	1.62
1.3	1.4	1.5	
1.6	1.9	1.6	
2.6	2.82		
		Fasted 24 hrs.	
		6.5	7.6
		13.2	16.0
		Fasted 48 hrs.	
		5.2	5.9
		8.0	9.2
		Fasted 76 hrs.	
		6.7	
		25.7	

Values in the mouse and rat were higher and more variable than in other species, and showed the rapid and large increases on fasting previously reported for the rat (8). The guinea pig showed a much lower response to fasting.

The plasma showed slight but consistently larger concentrations than in whole blood, in contrast to the findings in man of Stark and Somogyi (9). While this could possibly be due to the presence of an inhibitor in the cellular elements, no difficulty was encountered in the recovery of acetoacetic acid added to whole blood.

It was found that the known compounds giving absorption at 640 to 650 mμ were all destroyed by allowing 5 cc. of the filtrate acidified with 0.5 cc. of 5 N H_2SO_4 to stand with 0.8 cc. of 4 per cent $NaNO_2$ for 10 minutes at room temperature, or by brief heating. The red color produced by certain amino compounds is not diminished by this treatment, but usually increases. The majority of the filtrates were submitted to this treatment to determine whether the red color normally present affected the readings at 640 to 650 mμ. In all cases in which the filtrates were subjected to nitrous acid the green color was abolished, while a slight but variable residue of red remained. These filtrates when read at 640 to 650 mμ gave values as low as or lower than the reagent blank.

Pyruvic Acid

The amount of color absorption at 640 to 650 mμ that remained in the acidified and heated filtrates was investigated as a possible index of pyruvic acid content.

It was first found that pyruvic acid solutions were affected by standing at room temperature with the alkaline buffer, so that precautions were needed to have the buffers and the filtrates cold and to avoid long standing.

It was also found that pyruvic acid was more susceptible than acetoacetic acid to inhibitors of the diazo reaction, particularly copper, present in the reagents. This can be detected in the calibration curve, 5 to 80 γ being used; the lowest amount, 5 γ, will be largely inhibited. For the standards, pyruvic acid was redistilled *in vacuo* and kept in the frozen state at −8°.

Having obtained satisfactory calibration curves, we found, in accordance with Bueding and Wortis (10), who employed the hydrazone technique, that the pyruvic acid content of whole blood decreases on standing. It was not desirable to use iodoacetate, recommended by these authors as a stabilizing agent, as this compound affected the color intensities in our procedure.

Bueding and Wortis report satisfactory pyruvic acid determinations upon cerebrospinal fluid; we have likewise found that reproducible results upon blood plasma could be obtained, with good recovery of added pyruvate.

The blood samples were immediately chilled in ice water, and briefly centrifuged while cold. Whether any changes in plasma pyruvate occurred during this procedure is not known. The results of experiments upon the recovery of pyruvate added to plasma of several species are shown in Table III. These experiments illustrate that under certain conditions it may be possible to obtain simultaneous values for acetoacetic and pyruvic acids with the procedure.

Satisfactory recovery has not occurred following the addition of pyruvic acid to whole blood, and for this reason no results upon whole blood are reported.

TABLE III

Recovery of Pyruvic Acid Added to Plasma of Several Species

Animal	Pyruvic acid added	Pyruvic content	Animal	Pyruvic acid added	Pyruvic content
	γ per cc.	*γ per cc.*		*γ per cc.*	*γ per cc.*
Guinea pig		23	Rabbit		19.3
	10	31.5		10	29
	20	39		20	38.5
	30	50		30	49.5
Rat 1		17.8	Human 1		11
	10	27.5		20	32.5
	30	46.7	" 2		11.5
" 2		19.2		20	30
	10	30	" 3		8
	20	37		13.3	19
	30	45.5	" 4		13
			" 5		18

SUMMARY

A colorimetric method for the estimation of acetoacetic and certain closely related β-keto acids in small quantities of blood is described.

Under certain conditions a simultaneous estimate of the pyruvic acid content may be obtained from the procedure.

The method in its present form is not applicable to tissues or urine.

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THE SYNTHESIS OF GLUTATHIONE IN ISOLATED LIVER*

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The mechanism which is responsible for the formation of individual peptide bonds in biological systems has so far remained obscure. There has as yet been no successful demonstration of an *in vitro* enzymatic synthesis of a simple peptide composed of natural amino acids. Various investigators have approached this problem by employing model substances which contain a CO—NH linkage but differ from natural peptides in that either the carboxylic or amino component or both are not α-amino acids. This is true for the acyl peptides studied by Bergmann and Behrens (1) and also for acetylsulfanilamide (2), *p*-aminohippuric acid (3), hippuric acid (4), and glutamine (5). In all these cases the enzymatic formation of peptide bonds has been demonstrated. The incorporation of amino acids into the proteins of isolated tissues has recently been investigated with the aid of isotopic tracers (6–9), but experiments of this type do not lend themselves readily to a study of the mechanisms involved in the formation of individual peptide bonds.

We have previously reported the formation of glutathione from its constituent amino acids in rat liver slices (10). It was felt that glutathione was particularly suited to the study of peptide bond synthesis, because no other peptide is obtainable with comparable ease and purity from small amounts of animal tissues. These experiments dealing with the *in vitro* synthesis of glutathione were originally undertaken to test the hypothesis that *N*-acetylamino acids which appear to be intermediates in the normal metabolism of amino acids (11) might be concerned in peptide synthesis (12–14). Experiments with liver slices yielded inconclusive results in that glycine and *N*-acetylglycine were utilized equally well for glutathione formation.

In the course of attempts to differentiate between the behavior of the free amino acid and its acetyl derivative it was observed that glutathione synthesis proceeds readily in liver homogenates. In this system acetylglycine, in contrast to glycine, proved to be ineffective. This report describes some experiments which have been carried out to study the con-

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ditions associated with the formation of glutathione in these broken cell preparations.

EXPERIMENTAL

Synthesis of Isotopic Amino Acids—Glycine and DL-glutamic acid labeled with N^{15} were prepared as described by Schoenheimer and Ratner (15). The amino acids contained 31.5 atom per cent excess N^{15}.

C^{14}-Glycine—Carboxyl-labeled glycine was synthesized by the following series of reactions:

$$CH_3C^{14}OOK \xrightarrow{C_6H_5COBr} CH_3C^{14}OBr \quad (1)$$

$$CH_3C^{14}OBr + Br_2 \longrightarrow BrCH_2C^{14}OBr + HBr \quad (2)$$

$$BrCH_2C^{14}OBr + H_2O \longrightarrow BrCH_2C^{14}OOH + HBr \quad (3)$$

$$BrCH_2C^{14}OOH + NH_3 \longrightarrow NH_2CH_2C^{14}OOH + HBr \quad (4)$$

$CH_3C^{14}OOH$ was prepared by the interaction of CH_3MgBr with $C^{14}O_2$ Anhydrous potassium acetate was heated with benzoyl bromide in the presence of benzoic acid to yield acetyl bromide as described by Anker (16). The acetyl bromide was collected in an ice-cooled flask and 1.1 moles of bromine per mole of acetyl bromide were added slowly. The mixture was warmed and heated on a steam bath for 1½ hours and then freed of excess bromine and of HBr by a stream of nitrogen. An excess of water was added dropwise to the ice-cooled bromoacetyl bromide. The clear aqueous solution of bromoacetic acid was then added to a volume of concentrated NH_3 containing 70 M excess. The solution was kept at room temperature for several hours and then evaporated *in vacuo* to dryness. The residue was dissolved in a small volume of water and the glycine precipitated by addition of 4 volumes of methanol. The yield after one recrystallization from water-ethanol was 55 to 60 per cent, based on the potassium acetate used. The radioactivity of the glycine was 100,000 counts per minute counted as an infinitely thick sample after conversion to $BaCO_3$.

This method for the preparation of carbon-labeled glycine has been found to be more convenient and to give higher yields than that described in the literature (17). Little handling of the radioactive intermediates is necessary and after the distillation of acetyl bromide all further operations can be carried out in the same flask.

Acetylglycine was prepared either from C^{14}-glycine or N^{15}-glycine as previously described (18).

Incubation Experiments—In the experiments with intact tissue (Table I, Experiments 1 to 3) 1.5 gm. of liver slices were suspended in a medium of the following composition: labeled glycine or acetylglycine 0.01 M, Krebs' phosphate buffer of pH 7.4, and 25 mg. of carrier glutathione. The total volume per flask was 20 ml.

The liver homogenates were prepared by dispersing pigeon liver in a Waring blendor in a medium of the following composition: phosphate buffer of pH 7.4 0.05 M, KCl 0.03 M, $MgSO_4$ 0.0024 M, glutamic acid 0.01 M, cysteine 0.003 M, C^{14}-glycine 0.016 M, and 25 mg. of carrier glutathione. The homogenates contained 1.6 gm. of pigeon liver in a total volume of 20 ml. The final pH was 7.4, and the time of incubation 1 hour.

The medium in Experiment 1, Table IV, contained N^{15}-glutamic acid instead of non-isotopic glutamic acid. The molar ratio of C^{14}-glycine to N^{15}-glutamic acid was 1:1. In Experiment 2, Table IV, there were added C^{14}-glycine 0.016 M, N^{15}-NH_4Cl 0.008 M, and non-isotopic glutamic acid 0.016 M.

Isolation of Glutathione—25 mg. of non-isotopic glutathione per flask were added to the incubation medium in order to facilitate the isolation of isotopic glutathione in quantities sufficient for purification and isotope analysis. No differences in the isotope concentration of glutathione were observed whether the carrier was added before or after incubation. After incubation the reaction mixture was deproteinized with trichloroacetic acid and glutathione was precipitated first as the cadmium salt and then as the cuprous mercaptide as described by Waelsch and Rittenberg (19). The purity of the glutathione samples which were obtained from the experiments with amino acids labeled by N^{15} was checked by determination of Kjeldahl nitrogen. The majority of the glutathione samples which contained C^{14} were redissolved by the addition of an excess of cuprous oxide and reprecipitated by aeration (20). This treatment did not change the isotope concentration in the mercaptides, indicating that the product obtained in the first precipitation with cuprous oxide was pure. Reprecipitation of glutathione containing C^{14} in the presence of non-isotopic glycine likewise failed to depress the isotope concentration.

Glutathione formed in homogenates in the presence of C^{14}-glycine contained isotopic carbon only in the glycine moiety, as shown by the following experiment. 100 mg. of glutathione, with a C^{14} content of 151 counts per minute as an infinitely thick sample after combustion to barium carbonate, were hydrolyzed by refluxing with 20 per cent HCl for 8 hours. Glutamic acid was isolated from the hydrolysate as the hydrochloride, cysteine as the cuprous mercaptide, and glycine in the form of its toluenesulfonyl derivative. No radioactivity was detectable in the glutamic acid and in cysteine. The C^{14} content of the toluenesulfonylglycine was 171 counts per minute as compared to 167 counts calculated from the C^{14} content of glutathione. The radioactivity of glutathione therefore resided exclusively in the glycine moiety of the peptide.

The quantity of glutathione (in mg.) which is newly synthesized (x)

can be calculated from the specific activity (C_0) of the C^{14}-glycine added, the specific activity (C) of glycine carbon in the isolated glutathione, and the sum of glutathione (in mg.) originally present in the tissue (G_1) and carrier glutathione added (G_2).

$$x = \frac{C \times (G_1 + G_2)}{C_0} \tag{1}$$

This involves the assumption that the added isotopic glycine is not significantly diluted by glycine from the tissues. That this is the case is shown by the observation that doubling the molarity of isotopic glycine in the incubation medium did not raise the isotope concentration of glutathione. Since the amount of carrier glutathione was always much larger (10 to 15 times) than the tissue glutathione, fluctuation in the original glutathione content of the tissues will introduce only a small error in the calculation of x. Glutathione determined iodometrically (21) in aliquot samples of the tissue used for incubation was found to vary from 1.3 to 1.7 mg. per gm. of wet tissue. An average value of 1.5 mg. for G_1 was used for the calculation of the quantities of newly synthesized glutathione according to equation (1).

No attempt has been made to determine in the present experiments whether the total quantity of glutathione increases. It is conceivable that concurrently with its formation glutathione is also being removed and in this case the total quantity of glutathione may remain unchanged during the experimental period. The possibility that there is a considerable net decrease of glutathione during incubation of the homogenates under our conditions has been ruled out by the finding that the isotope concentrations in glutathione are the same whether the carrier glutathione is added before or after incubation[1] and by glutathione determinations in controls before and after incubation.

Isotope Analyses—The amino acids and glutathione samples which contained N^{15} were digested by the Kjeldahl procedure and the ammonia converted to nitrogen for mass spectrometric analysis as described by Rittenberg *et al.* (22). For C^{14} analysis the compounds were burned in a micro combustion apparatus and carbon dioxide precipitated as barium carbonate. The C^{14} content of the barium carbonate samples was measured with a thin window Geiger-Müller counter by the procedure of Reid (23). Samples were counted for a sufficient length of time to insure less than 5 per cent probable error. The C^{14} values are given, unless stated otherwise, as counts of C^{14} per minute of $BaCO_3$ samples corrected for infinite thickness.

[1] Unpublished results by R. B. Johnston.

DISCUSSION

It has been shown previously that in rat liver slices isotopic nitrogen from labeled glycine and *N*-acetylglycine is incorporated into glutathione at a similar rate (10). On the basis of these results it could not be decided whether acetylation of the amino acid was a preliminary step in the formation of glutathione. Equivocal results are to be expected from experiments with liver slices, since both the acetylation of amino acids and the hydrolysis of the acetyl derivatives take place under these conditions (11). That acetylation does not precede the incorporation of glycine into peptide linkages is suggested by experiments of shorter duration in which glycine gave rise to significantly higher isotope concentrations in glutathione than did acetylglycine (Table I). In these cases acetylglycine is evidently not used as such but only after conversion to the free amino acid. The rate of splitting of the acetyl derivative therefore appears to be slightly slower than the rate of glycine entrance into glutathione.[2] This difference in reactivity of glycine and acetylglycine is accentuated when

TABLE I

Aerobic Formation of Glutathione from Labeled Amino Acids in Isolated Liver

The composition of the incubation medium is described in the experimental part.

Experiment No.	Tissue	Time	Relative isotope concentration in glutathione*	
			Labeled glycine added	Labeled acetylglycine added
		hrs.		
1	Rat liver slices	½	0.64	0.22
	" " "	1	1.04	0.90
2	" " "	½	1.76	1.2
3	Pigeon liver slices	1	0.52	1.4
4	" " homogenates	1	1.89	0.23
5	" " "	1	1.89	0.15
	" " "	1	1.7	0.06

* Atom per cent excess N^{15} or specific activity (C^{14}) in glycine moiety calculated for 100 atom per cent excess N^{15} and a specific activity of 100 respectively in labeled amino acid added. In Experiments 1 to 4 the amino acids were labeled by N^{15}, in Experiment 5 by C^{14}.

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[2] In one experiment with pigeon liver slices considerably more isotope was incorporated into glutathione from acetylglycine than from glycine (Experiment 3, Table I). Because of the results obtained subsequently with homogenates, this observation was not further investigated.

the same process is studied in pigeon liver homogenates. In this system the utilization of acetylglycine for glutathione synthesis is very small (Table I, Experiments 4 and 5) while isotopic glycine is incorporated at a rate comparable to that occurring in intact slices. It is evident therefore that in the formation of the cysteinylglycine moiety of glutathione *N*-acetylglycine is not an intermediate and that in this case at least the *N*-acetylamino acid does not take part in the formation of the peptide bond. Results by Simmonds, Tatum, and Fruton (24) on the utilization of acetylamino acids by *Neurospora* mutants and those by Cohen and McGilvery (3) on the formation of *p*-aminohippuric acid have led these authors to the same conclusion. It is worthy of note that the enzyme system which converts the *N*-acetyl derivative to the free amino acid appears to be almost completely inactivated by homogenization of the liver tissue.

As is shown by the data in Table I the rate of aerobic glutathione synthesis in pigeon liver homogenates, measured by the incorporation of isotopic glycine, is of the same order of magnitude as that in intact slices. While the variations in duplicate experiments with aliquots of the same liver homogenate never exceeded 10 per cent, the rate of glutathione synthesis was found to fluctuate considerably with tissue from different animals. A total of fourteen experiments was carried out to determine the formation of glutathione under aerobic conditions. In nine of these the newly synthesized glutathione, calculated from the incorporation of glycine carbon, amounted to 0.2 to 0.4 mg. per gm. of liver per hour. The rate of synthesis was materially lower in three experiments (0.04, 0.08, and 0.09 mg.) and greater (0.75 and 1.5 mg.) in two cases. Since changes in the quantities of glutathione during incubation were not determined, it is not possible to state whether the incorporation of isotopic carbon results in an increase in the total quantity of the tripeptide.

The stimulating effect of adenosine triphosphate on the rate of glutathione formation is shown in Tables II and III and Fig. 1. The acceleration by adenosine triphosphate is optimal at a molarity of 5×10^{-4} and is reversed at higher concentrations. The data indicate a participation of adenosine triphosphate in the formation of the peptide linkages in glutathione, though it is not clear in what manner adenosine triphosphate enters into the synthetic process. 2,4-Dinitrophenol has been reported to block phosphorylations without affecting respiration (25–27). In accord with these findings, dinitrophenol was found to interfere markedly with glutathione formation. It should be noted, however, that the inhibitor was effective only in relatively high concentrations (4×10^{-4} M). The results obtained on addition of succinate, fumarate, or malonate (Table III) confirm the impression that the entrance of glycine into peptide linkage is associated with energy-yielding reactions.

TABLE II

Effect of Adenosine Triphosphate on Incorporation of C^{14}-Glycine into Glutathione in Pigeon Liver Homogenates

Experiment No.	Gas phase	ATP added	C^{14} in glutathione	Glutathione synthesized per hr.
		M	*counts per min.*	γ
I	O_2		46, 47	62, 64
	N_2		11	15
	"	1×10^{-3}	38, 33	51, 46
II	O_2		127	180
	N_2		6.5, 3.7	9, 5
	"	1×10^{-3}	33, 30	46, 41
III	N_2		24	32
	"	1×10^{-3}	64	86
IV	O_2		416	670
	N_2		65	106
	"	5×10^{-4}	110	179
V	O_2		235	381
	"	5×10^{-4}	782	1267
	N_2		140	227
	"	5×10^{-4}	132	214
VI	O_2		858	1390
	"	7×10^{-4}	1656	2683
	N_2		230	372
	"	7×10^{-4}	297	481

Each flask contained 1.6 gm. of liver, amino acids, and buffer as described in the experimental part; total volume per flask, 20 ml.; incubated for 1 hour at 37°.

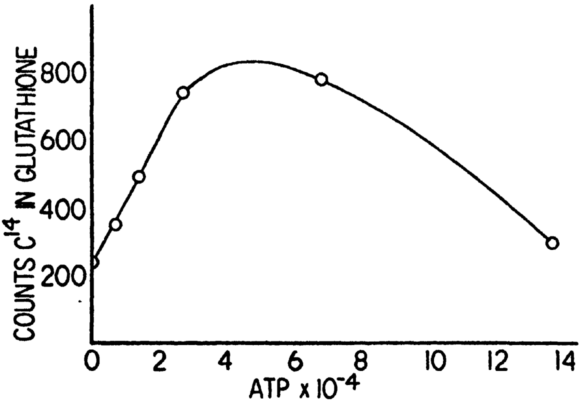

FIG. 1. Effect of adenosine triphosphate on the incorporation of C^{14}-glycine into glutathione in pigeon liver homogenates. Each flask contained 1.6 gm. of liver, amino acids, and buffer as described in the experimental part; total volume per flask, 20 ml.; incubated for 1 hour in oxygen at 37°.

The incorporation of glycine into glutathione is reduced to much lower, though still significant, levels under anaerobic conditions. In eight experiments in which glutathione formation in the presence of oxygen and in nitrogen was compared, the average value for anaerobic synthesis was 17 per cent of that found under the aerobic conditions. In a number of experiments the level of anaerobic synthesis was substantially raised by the addition of adenosine triphosphate. However, it was not possible to reproduce this effect with regularity. Under anaerobic conditions adenosine triphosphate was without effect in two experiments out of six (Table II) and was sometimes inhibitory at higher concentrations.

TABLE III

Effect of Metabolites and Inhibitors on Incorporation of C^{14}-Glycine into Glutathione in Pigeon Liver Homogenates

1.6 gm. of rat liver per flask. Total volume, 20 ml.; composition of medium as described in the experimental part; incubated at 37° in oxygen for 1 hour.

Additions	Molarity	C^{14} in glutathione*
		counts per min.
None		100
Adenosine triphosphate	3×10^{-4}	313
Succinate	1×10^{-2}	264
Fumarate	1×10^{-2}	250
Ammonium chloride	1×10^{-2}	77
Glutamic acid omitted		25
Cysteine omitted		33
Malonate	1×10^{-2}	39
2,4-Dinitrophenol	1×10^{-4}	105
"	4×10^{-4}	10

* Since the activities of different homogenates varied considerably, the data, which are taken from different series, were recalculated for a relative activity of 100 counts of C^{14} in the control experiment.

The reason for the occasional failure of adenosine triphosphate to stimulate glutathione synthesis anaerobically has so far remained obscure. The synthesis of glutathione from the constituent amino acids must involve at least two steps and it is conceivable that only one of these is dependent on the supply of phosphate bond energy. The effect of adenosine triphosphate may therefore become evident only when the supply of other endogenous factors is adequate for the synthesis of the entire molecule.

The majority of the experiments which are reported here have been concerned with the incorporation of glycine into glutathione, but evidence has also been obtained to indicate that the processes under investigation include a replacement of glutamic acid residues in the tripeptide. The data in Table IV show the incorporation into glutathione of C^{14} and N^{15}

from a medium in which C^{14}-glycine and N^{15}-DL-glutamic acid were present in equimolar quantities. Under these conditions the uptake of glycine carbon into glutathione is more than twice that of nitrogen from glutamic acid. Since the isotopic glutamic acid used was in the racemic form, and since it can be assumed that only the L isomer is directly employed in peptide synthesis, the molar concentration of glutamic acid was in effect only one-half that of glycine. Moreover, the liver preparation presumably contains glutamic acid dehydrogenase (28) and a replacement of the isotopic nitrogen in glutamic acid by ordinary nitrogen would be expected to result from the action of this enzyme. In this case the N^{15} concentration in glutathione would not be a true measure of the rate of entrance of glutamic acid into the tripeptide. The reversible deamination of glutamic acid under these conditions becomes evident from an experiment with $N^{15}H_4Cl$ in addition to C^{14}-glycine and non-isotopic glutamic acid. Glutamic acid was isolated after hydrolysis of glutathione and found to contain roughly the same isotope concentration as in the experiment with added N^{15}-glutamic acid, showing that amination of ketoglutaric acid must have occurred.

TABLE IV

Formation of Glutathione in Pigeon Liver Homogenates

Isotopic additions	Relative isotope concentrations in glutathione	
	C^{14}*	N^{15}†
C^{14}-Glycine and N^{15}-glutamic acid	1.40	0.60
	1.34	0.43
" and N^{15}-ammonium chloride	1.76	0.48

* Specific activity of glycine moiety calculated for a specific activity of 100 in the glycine added.

† Atom per cent excess N^{15} in glutamic acid moiety calculated for 100 atom per cent excess N^{15} in glutamic acid or NH_4Cl added. Each flask contained 1.6 gm. of liver, amino acids, and buffer as described in the experimental part; total volume per flask, 20 ml.; incubated for 1 hour in oxygen at 37°.

It is also possible that the introduction of N^{15} from labeled ammonia observed here is the result of a reversible deamination of the glutamyl residue in glutathione itself. Nevertheless it appears likely that the process under investigation involves the renewal of both the glutamylcysteine and cysteinylglycine linkages in the glutathione molecule.

SUMMARY

1. Incubation of liver slices and homogenates in the presence of C^{14}-glycine or N^{15}-glutamic acid results in the formation of labeled glutathione, demonstrating the synthesis of the tripeptide under *in vitro* conditions.

2. *N*-Acetylglycine is utilized for glutathione formation in liver slices but not in liver homogenates. This eliminates the acetyl derivative as an intermediate in the synthetic process.

3. Adenosine triphosphate markedly accelerates the aerobic synthesis of glutathione in liver homogenates. This stimulation by adenosine triphosphate was observed also under anaerobic conditions but could not always be reproduced.

4. A method for the synthesis of glycine labeled by isotopic carbon is described.

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A STUDY OF THE CHEMICAL ORIGINS OF GLYCOGEN BY USE OF C^{14}-LABELED CARBON DIOXIDE, ACETATE, AND PYRUVATE*

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The problem of the source of the carbons of liver glycogen has commanded the attention of biochemists for several years (1, 2). It is the purpose of the present paper to provide quantitative data on the relative proportions of the glycogen carbons which were originally the α-carbons of pyruvate molecules.

Following the demonstration by Lardy and Ziegler (3) that phosphorylation of pyruvate to phosphopyruvate can proceed directly in the presence of potassium ions, the necessity for postulating the formation of phosphomalate and phosphooxalacetate as intermediates in the formation of phosphopyruvate has been removed (4, 5). However, the question of the relative proportion of pyruvate molecules which enter the dicarboxylic acid shuttle before phosphorylation compared with those which are phosphorylated directly has not been previously determined. Our experiments provide evidence on this question. However, they shed no light on the question of whether phosphorylation of dicarboxylic acids can or does occur.

If one employs α-labeled pyruvate ($CH_3C^{14}OCOOH$) as substrate and determines the position and relative concentration of C^{14} in the glucose units of glycogen, one would expect to find C^{14} concentrated in C-2 and C-5 of the glucose carbon chain if pyruvate molecules are phosphorylated directly. If C^{14} should be symmetrically placed in C-1, C-2, C-5, and C-6, then one may conclude that half of the α-C-labeled oxalacetate had been converted into β-C-labeled oxalacetate through equilibration with the symmetrical dicarboxylic acids, fumaric and succinic.

The experiments to be described provide evidence that carboxylation of pyruvate and equilibration of oxalacetate with the symmetrical dicarboxylic acids do occur more rapidly than does direct phosphorylation of pyruvate.

EXPERIMENTAL

Labeled glycogen was prepared by incubating rabbit liver slices *in vitro* in a potassium-rich medium with pyruvate as substrate according to the

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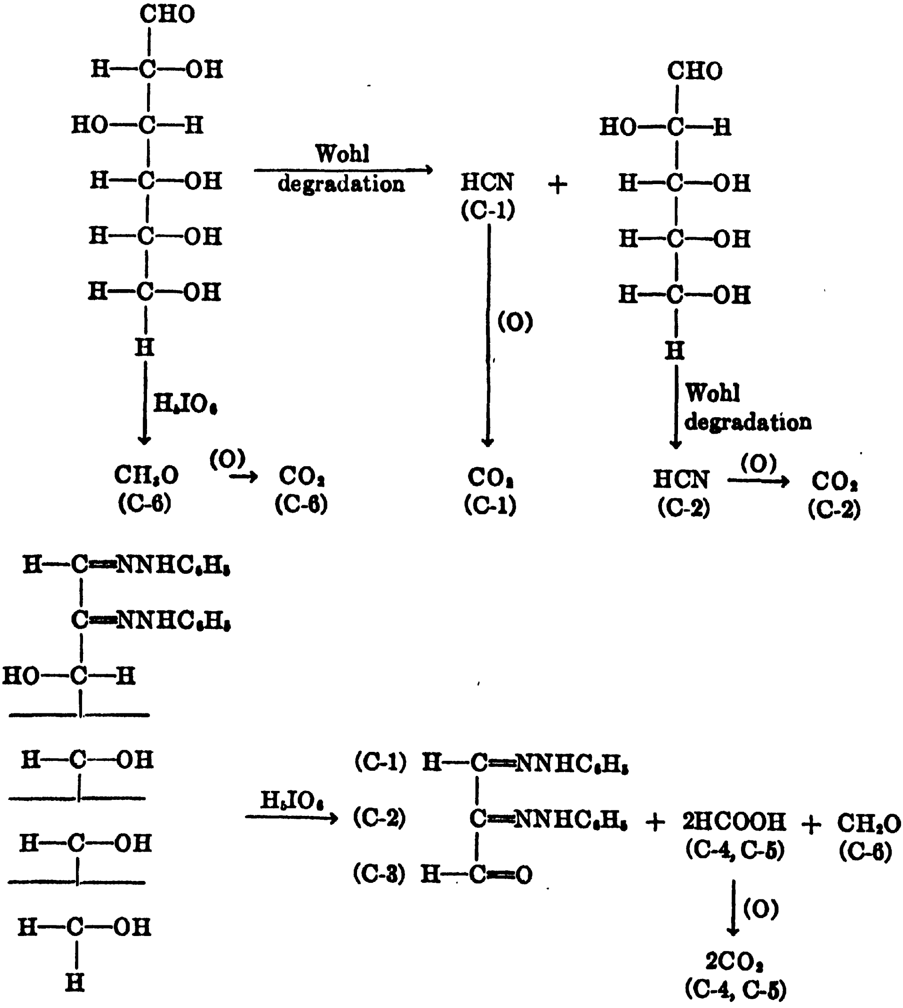
method previously described (6). Experiments were carried out in the presence of $NaHC^{14}O_3$, carboxyl-labeled acetate ($CH_3C^{14}OOH$), and carbonyl-labeled pyruvate ($CH_3C^{14}OCOOH$).

General Scheme for Degradation of Glucose



Wood, Lifson, and Lorber (7), in experiments on the synthesis of liver glycogen by rats, developed a method for degrading glucose in order to determine the position of the isotopic carbon in the molecule. For the present investigation, the mode of degradation shown in the accompanying diagram was developed and used.

Hydrolysis of Glycogen—In a small flask fitted with a reflux condenser, 420 mg. of glycogen suspended in 3 ml. of 1 N sulfuric acid were heated at 100° for 2 hours. The solution was then cooled, diluted with 5 ml. of water, and neutralized with a saturated barium hydroxide solution. The supernatant liquid obtained after centrifugation was analyzed for its glucose content according to the method of Folin (8).

Two Successive Wohl Degradations of Glucose; Isolation of C-1 and C-2—An aliquot of the glucose solution (glycogen hydrolysate) containing 300 mg. of glucose was evaporated to dryness *in vacuo*. To the residue was added 0.22 gm. of hydroxylamine acetate plus 0.40 ml. of glacial acetic acid, and the mixture was heated at 100° for 15 minutes. After cooling, 0.25 ml. of acetic anhydride was added to the solution; heating at 100° was resumed for 18 minutes. When the solution had been cooled to room temperature, it was seeded with gluconitrile; at the end of 12 hours the precipitate was filtered by suction, washed four times with 0.1 ml. portions of a 2:1 acetone-glacial acetic acid solution, four times with 0.1 ml. portions of a 9:1 acetone-glacial acetic solution, similarly with acetone, and finally with ether. 80 mg. of gluconitrile were obtained, m.p. 146°.

80 mg. of gluconitrile were dissolved in 3 ml. of water and nitrogen was passed through the resulting solution, which was maintained at 85° in a water bath; the emerging gases were bubbled into a solution of silver nitrate. Decomposition was complete in about 2 hours, as evidenced by cessation of precipitation of silver cyanide (C-1). The cyanide was converted to CO_2 and the radioactivity of C-1 determined as $BaC^{14}O_3$.

The aqueous solution containing the arabinose formed in the first degradation was evaporated to dryness *in vacuo*. The residue, which had crystallized after remaining in the vacuum desiccator for 24 hours, was converted to arabononitrile by using 0.05 gm. of hydroxylamine acetate, 0.05 ml. of glacial acetic acid, and 0.05 ml. of acetic anhydride, in sequence as described for gluconitrile. 2 ml. of water were added to the arabononitrile solution and decomposition was effected as described for gluconitrile. At the end of 7 hours, 9 mg. of silver cyanide containing C-2 were obtained. The cyanide was converted to CO_2 and the radioactivity of C-2 determined as $BaC^{14}O_3$.

Isolation of C-6—18 mg. of glucose, contained in an aliquot of the glycogen hydrolysate solution, were oxidized according to the procedure described by Reevés (9). However, instead of isolating the formaldehyde as the dimedon derivative, the following method was employed. When the yellow color had disappeared after the addition of the sodium arsenite reagent, the solution was made alkaline to phenol red by the addition of 1 N sodium hydroxide, and was distilled until crystals began to form in the distillation flask; the distillate was cooled in an ice-salt bath. Then 0.5 gm. of potassium permanganate was added to the distillate, and the latter was refluxed while a slow stream of nitrogen was bubbled through the solution. The emerging gases were passed into a solution of saturated barium hydroxide. 5 to 10 mg. of barium carbonate were obtained from the carbon dioxide, which, in turn, had been derived from the formaldehyde, representing C-6. The radioactivity of C-6 was determined by counting the $BaC^{14}O_3$.

Oxidation of Glucosazone—The osazone of the labeled glucose was prepared by the usual procedure and counted as such. A portion was then oxidized to the 3-carbon osazone by periodate. This oxidation, as originally described (10), was carried out in acid solution. However, it was desirable, in this case, to carry out the reaction in alkaline solution, a condition under which formaldehyde is obtained quantitatively from glucose (9). In this way the formic acid, representing C-4 and C-5, would not be contaminated with material derived from the oxidation of formaldehyde (C-6).

50 mg. of glucosazone were dissolved in 20 ml. of 66 per cent ethanol, the solution was cooled to 30°, 1.38 ml. of 1 N sodium bicarbonate were added, and, finally, 1.38 ml. of 0.3 M paraperiodic acid reagent were introduced. An orange-yellow precipitate was formed almost immediately; after 15 minutes the mixture was centrifuged. The precipitate, 1,2-bisphenylhydrazone of mesoxalaldehyde, after being recrystallized from 66 per cent ethanol, was counted as such. To the filtrate containing formic acid derived from C-4 and C-5, 1.38 ml. of 1.2 M sodium arsenite solution and 2.1 ml. of 1 N hydrochloric acid were added, and after the red color had disappeared the solution was made slightly acid to Congo red by the addition of sodium hydroxide. Carbon dioxide was driven out of the system by passing a stream of nitrogen through the solution while the latter was refluxing, and then 1 gm. of mercuric oxide was added to oxidize formic acid to carbon dioxide (formaldehyde is not oxidized by mercuric oxide under similar conditions). The CO_2 thus obtained, representing C-4 and C-5, was counted as $BaC^{14}O_3$.

Results and Comment

Relative Radioactivities of Glucose Carbons from (a) $NaHC^{14}O_3$ and (b) $CH_3C^{14}OONa$—The results of the determinations of the radioactivities of the several carbon fractions derived from the labeled glucose containing C^{14} from $C^{14}O_2$ or $CH_3C^{14}OONa$ are presented in Table I. These data serve a twofold purpose: (1) the calculation of a conversion factor to permit the comparison of measurements of $BaC^{14}O_3$ and C^{14}-labeled osazones, and (2) the determination of the positions of the C^{14} in the glucose unit in these experiments.

If it were possible to isolate each carbon in the glucose molecule, the determination of their relative activities would be straightforward. However, in the degradation described, only C-1, C-2, and C-6 are counted individually in the form of $BaCO_3$. The activities of C-3, C-4, and C-5, on the other hand, have been calculated from the measurements of C-1 and C-2 as $BaC^{14}O_3$, of C-1 through C-6 as glucosazone, and of C-1 through C-3 as 1,2-bisphenylhydrazone of mesoxalaldehyde.

The two osazones have almost identical average atomic numbers; consequently, their activities should be directly comparable. $BaCO_3$ has

TABLE I

Radioactivity[1] of Fractions Obtained from Degradation of Glucose

Experiment No.	Substrate	Fraction	Counts per min. per mM compound
1	$C^{14}O_2 + CH_3COCOOH$	A. Glucosazone	16,198
		B. 3-Carbon osazone	8,332
		C. ($BaCO_3$) C-1	0
		D. " C-2	0
		E. " C-4,C-5	5,772
		F. " C-6	0
		G. $BaCO_3$ from glucose	3,665
2	$C^{14}O_2 + CH_3COCOOH$	A. Glucosazone	16,204
		B. 3-Carbon osazone	8,036
		C. ($BaCO_3$) C-1	0
		D. " C-2	0
		E. " C-4,C-5	5,392
		F. " C-6	0
		G. $BaCO_3$ from glucose	3,570
3	$CH_3C^{14}OONa + CH_3COCOOH$	A. Glucosazone	7,380
		B. 3-Carbon osazone	3,870
		C. ($BaCO_3$) C-1	0
		D. " C-2	0
		E. " C-4,C-5	2,496
		F. " C-6	0
		G. $BaCO_3$ from glucose	1,650

[1] The probable error in individual counts is 2 per cent.

a much higher average atomic number than the osazones; consequently, the former will produce more back-scattering and will have a higher apparent activity. Therefore, in order to compare the activities of the various $BaCO_3$ fractions with those of the osazones, a conversion factor was experimentally determined. Glycogen was prepared by incubating rabbit liver slices with pyruvate in the presence of $C^{14}O_2$ or $CH_3C^{14}OONa$. The glucose containing isotopic carbon, derived from the glycogen, was degraded and comparison made between the osazone and $BaCO_3$ activities. The results of our experiments are shown in Table II. On the premise that isotopic carbon is equally distributed between C-3 and C-4 of the glucose molecule (7), the activity per mM of glucosazone Fraction A should be twice that of the 3-carbon osazone Fraction B; moreover, the activity of the C-4,C-5 Fraction E should be 3/2 that of the $BaCO_3$ Fraction G, derived from the combustion of glucose. As shown in the second and third columns of Table II, these relations were found to obtain.

Although the activities of the two osazones were directly comparable, as were those of the various $BaCO_3$ fractions, the former were not directly comparable with the latter. Thus, if the degree of back-scattering of the osazones was the same as that of $BaCO_3$, Fraction B would be twice Fraction E. However, the $BaCO_3$ activities exceed those predicted from the osazone activities by approximately 34 per cent (Column 4, Table II). In subsequent experiments with carboxyl-labeled pyruvate, the osazone counts have, therefore, been multiplied by 1.34 in computing the relative activities of the glycogen carbons in order to make them comparable with the $BaCO_3$ counts.

TABLE II

Interrelationships of Activities of Various Fractions

Experiment No.	$\frac{\text{Fraction A}}{\text{Fraction B}}$	$\frac{\text{Fraction E}}{\text{Fraction G}}$	$\frac{2 \times \text{Fraction E}}{\text{Fraction B}}$
1	1.94	1.57	1.38
2	2.02	1.51	1.34
3	1.91	1.51	1.29

A sample calculation from Experiment 4 follows.

Direct Activity Measurements of C-1, C-2, and C-6

	counts per min. per mM
C-1 ($BaCO_3$)	254 (C-1)
C-2 "	273 (C-2)
C-6 "	265 (C-6)

Calculation of Activities of C-3, C-4, and C-5

counts per min. per mM

1. Measured activity of 3-carbon osazone 629

   Activity of 3-carbon fraction calculated from glucosazone, $\frac{1424}{2}$ = 712

   Average activity, $\frac{629 + 712}{2}$ = 670

2. 3-Carbon fraction activity corrected to $BaCO_3$ activity, 670 × 1.34 = 898
3. Activity of C-3 =

   3-Carbon fraction activity minus (C-1 activity + C-2 activity) =

   898 − (254 + 273) = 371 (C-3)

	counts per min. per mM
4. Assume activity of C-4 = activity of C-3	= 371 (C-4)
5. Average measured activity of C-4 + C-5	= 350
Activity of C-4 + C-5, 350 × 2	= 700
" " C-5, 700 − 371	= 329 (C-5)

From the measured and calculated activities of C-1 through C-6, the relative C^{14} activities of the glucose carbons may be calculated.

The data in Table I indicate that the C^{14} incorporated in the glucose molecule, prepared *in vitro* as described, and derived from $C^{14}O_2$ or from $CH_3C^{14}OONa$, is present in C-3 and C-4, is not detectable in C-1, C-2, and C-6, and is probably not present in C-5. Wood *et al.* (7, 11), using $C^{13}O_2$ and $CH_3C^{13}OONa$ *in vivo*, have reported that only glucose carbons 3 and 4 contained isotopic carbon, although the distribution of the isotope between these two positions was not indicated unequivocally. In a recent foot-note, Lifson, Lorber, Sakami, and Wood (12) have stated that, in glycogen produced *in vivo*, a trace (1 to 2 per cent) of $C^{14}O_2$ does appear in carbons 1, 2, 5, and 6.

Relative Radioactivities of Glucose Carbons from $CH_3C^{14}OCOONa$—The data on the distribution of isotopic carbon in glucose which have been derived from $CH_3C^{14}OCOOH$ are given in Table III. It is to be noted that, with carbonyl-labeled pyruvate ($CH_3C^{14}OCOOH$) as substrate, isotopic carbon is found in all 6 carbons of the glucose unit of glycogen. It is also found that the isotopic carbons are symmetrically distributed between (C-1, C-2, and C-3) and (C-4, C-5, and C-6). This provides confirmatory evidence for the formation of the 6-carbon units of glycogen from two tautomeric 3-carbon fractions.

It is further to be noted that with carbonyl-labeled pyruvate as substrate the concentration of isotopic carbon in C-1 and C-6 of the glucose molecule is relatively high, though not as great as in C-2 and C-5. This indicates, on the one hand, that, as in the case of muscle extracts (3), the reaction, pyruvate → phosphopyruvate, occurs in rabbit liver slices, and, on the other hand, that the dicarboxylic acid shuttle is quantitatively a very important intermediate pathway.

It is also apparent, from the high concentration of C^{14} in C-3 and C-4, that considerable incorporation of $C^{14}O_2$, derived from the metabolism of $CH_3C^{14}OCOOH$, has occurred. This is to be interpreted as indicating (*a*) the conversion of carboxyl-labeled pyruvate to $C^{14}O_2$ through the tricarboxylic acid cycle, (*b*) the production of carboxyl-labeled pyruvate through the dicarboxylic acid shuttle, and (*c*) the conversion of the carboxyl-labeled pyruvate to glycogen by phosphorylation and a reversal of the glycolytic reactions.

The minimum number of α-labeled pyruvate molecules required to yield the approximate relative radioactivities observed has been estimated. For simplicity of calculation, the radioactivities of the glucose carbons have been rounded off to the nearest whole numbers (Table IV).

TABLE III

Relative Radioactivity of Glucose Carbons Derived from $CH_3C^{14}OCOOH$

Experiment No.	Fraction counted	Radioactivity, counts per min. per mM* compound	Position of C^{14} in glucose molecule	Relative isotope content in various positions of glucose molecule
4	Glucosazone	1424	C-1	1
	3-Carbon osazone	629	C-2	1.1
	($BaCO_3$) C-1	254	C-3	1.5
	" C-2	273	C-4	1.5
	" C-4,C-5	350	C-5	1.3
	" C-6	265	C-6	1.05
5	Glucosazone	1785	C-1	1.1
	3-Carbon osazone	851	C-2	1.6
	($BaCO_3$) C-1	292	C-3	1.7
	" C-2	430	C-4	1.7
	" C-4,C-5	445	C-5	1.7
	" C-6	261	C-6	1
6	Glucosazone	5341	C-1	1
	3-Carbon osazone	2487	C-2	1.45
	($BaCO_3$) C-1	827	C-3	1.6
	" C-2	1199	C-4	1.6
	" C-4,C-5	1300	C-5	1.4
	" C-6	865	C-6	1.05

* The probable error in individual counts is 4 to 5 per cent.

TABLE IV

Comparison of Relative Activities of Glucose Carbons, Found and Approximated

	Average relative activities found	Approximate relative activity	
C-1	1.0	1	2
C-2	1.4	1.5	3
C-3	1.6	1.5	3
C-4	1.6	1.5	3
C-5	1.5	1.5	3
C-6	1.0	1	2

Taking into consideration the various metabolic possibilities such as decarboxylation to acetate, carboxylation to oxalacetate, equilibrium with fumarate, oxidation to CO_2, and phosphorylation to phosphopyruvate, one obtains the following values for the minimum number of molecules of pyruvate involved. A minimum of 20 molecules of $CH_3C^{14}OCOOH$ would be required to provide isotopic carbon in C-3 and C-4, 8 molecules would have entered the dicarboxylic cycle as far as fumarate before being phosphorylated to phosphopyruvate, and 2 molecules would have been phosphorylated directly, making a total of 30 pyruvate molecules required to give the approximate relative activities listed above.

The detailed basis of these estimates is given below. α-C-Labeled pyruvate will be designated as $\overset{\beta}{\mathrm{C}}{-}\overset{\alpha}{\mathbf{C}}{-}\mathrm{C}$; β-C-labeled pyruvate, $\mathbf{C}{-}\mathrm{C}{-}\mathrm{C}$; carboxyl-labeled pyruvate, $\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}$; and the glucose unit as $\overset{1}{\mathrm{C}}{-}\overset{2}{\mathrm{C}}{-}\overset{3}{\mathrm{C}}{-}\overset{4}{\mathrm{C}}{-}\overset{5}{\mathrm{C}}{-}\overset{6}{\mathrm{C}}$. Isotopic carbons are designated $\mathbf{C}$.

1. By direct phosphorylation of $\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}$

$$2\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} + 2\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \longrightarrow \begin{array}{l} \mathrm{C}{-}\mathbf{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \\ \mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} \end{array}$$

2. Equilibration of $\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}$ in dicarboxylic acid shuttle before phosphorylation

$$8\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} \xrightarrow{\text{Shuttle}} \begin{array}{l} 4\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} \\ 4\mathbf{C}{-}\mathrm{C}{-}\mathrm{C} \end{array}$$

$$4\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} + 4\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \longrightarrow \begin{array}{l} 2\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \\ 2\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} \end{array}$$

$$4\mathbf{C}{-}\mathrm{C}{-}\mathrm{C} + 4\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \longrightarrow \begin{array}{l} 2\mathbf{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \\ 2\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathbf{C} \end{array}$$

3. (*a*) Conversion of $\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}$ to $\mathbf{C}\mathrm{O}_2 + \mathrm{CO}_2$
(*b*) Formation of $\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}$
(*c*) Direct phosphorylation of 20% of $\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}$
(*d*) Equilibration of 80% of $\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}$ before phosphorylation

(*a*) $20\mathrm{C}{-}\mathbf{C}{-}\mathrm{C} \rightarrow 20\mathbf{C}\mathrm{O}_2 + 20\mathrm{CO}_2$

(*b*) $20\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} + 20\mathbf{C}\mathrm{O}_2 \rightarrow$
$10\mathrm{C}{-}\mathrm{C}{-}\mathbf{C} + 10\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} + 10\mathbf{C}\mathrm{O}_2 + 10\mathrm{CO}_2$

(*c*) $$2\mathrm{C}{-}\mathrm{C}{-}\mathbf{C} + 2\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \longrightarrow \begin{array}{l} \mathrm{C}{-}\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \\ \mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}{-}\mathrm{C} \end{array}$$

(*d*) $$8\mathrm{C}{-}\mathrm{C}{-}\mathbf{C} \rightarrow \left\{\begin{array}{l} 4\mathrm{C}{-}\mathrm{C}{-}\mathbf{C} \\ 4\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \end{array}\right.$$

$$4\mathrm{C}{-}\mathrm{C}{-}\mathbf{C} + 4\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \longrightarrow \begin{array}{l} 2\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathrm{C} \\ 2\mathrm{C}{-}\mathrm{C}{-}\mathrm{C}{-}\mathbf{C}{-}\mathrm{C}{-}\mathrm{C} \end{array}$$

$$\begin{array}{ll} \textbf{Relative radioactivities of carbons} & \begin{array}{cccccc} \mathbf{2} & \mathbf{3} & \mathbf{3} & \mathbf{3} & \mathbf{3} & \mathbf{2} \end{array} \\ & \mathbf{C}{-}\mathbf{C}{-}\mathbf{C}{-}\mathbf{C}{-}\mathbf{C}{-}\mathbf{C} \\ \textbf{Carbon No. in glucose unit} & \begin{array}{cccccc} \mathbf{1} & \mathbf{2} & \mathbf{3} & \mathbf{4} & \mathbf{5} & \mathbf{6} \end{array} \end{array}$$

It would appear from the results presented that carboxylation of pyruvate to oxalacetate and equilibration in the dicarboxylic acid shuttle system occur about 4 times as fast as does direct phosphorylation of pyruvate molecules; and that about half as many pyruvate molecules are directly converted to glycogen as are converted first to CO_2 molecules, which subsequently appear in glycogen carbons 3 and 4.

The assistance of Miss Frances B. Nesbett in the preparation of the glycogen and of the Biophysical Laboratory of the Medical School for the radioactivity measurements is gratefully acknowledged.

SUMMARY

1. Chemical methods have been devised for the determination of the relative radioactivities of the carbon atoms comprising the glucose molecule.

2. Glycogen was made *in vitro* by rabbit liver slices with pyruvate as substrate in the presence of $C^{14}O_2$, $CH_3C^{14}OOH$, or $CH_3C^{14}OCOOH$.

3. In the presence of $C^{14}O_2$ and $CH_3C^{14}OOH$, only carbons 3 and 4 of the glucose unit of glycogen contained C^{14}.

4. In the presence of $CH_3C^{14}OCOOH$, all carbons of the glucose unit contained C^{14}.

5. The experimental results demonstrate the symmetry of carbons 1, 2, and 3 of the glucose molecule with respect to carbons 6, 5, and 4.

6. From the relative activities of the glucose carbons, the minimum number of labeled pyruvate molecules required to yield the experimental results has been estimated.

7. The proportion of pyruvate molecules which are (*a*) phosphorylated directly, (*b*) equilibrated with symmetrical dicarboxylic acids before phosphorylation, and (*c*) converted to $C^{14}O_2$ which is then combined with pyruvate with the production of carboxyl-labeled pyruvate has been estimated.

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PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

XIII. 3α,11α-DIHYDROXY-17α-STEROIDS*

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In addition to the compounds reported in the stepwise degradation of the side chain of 3α,11α-dihydroxycholanic acid (1), we obtained two new steroids possessing the 17α or unnatural configuration of the side chain. These were 3α,11α-dihydroxy-17α-pregnan-20-one and 3α,11α-dihydroxy-17α-etiocholanic acid. The normal configuration of the steroid side chain is considered 17β in keeping with the results of Sorkin and Reichstein (2), von Euw and Reichstein (3), and Gallagher and Long (4).

3α,11α-Dihydroxy-17α-pregnan-20-one was obtained by heating the diacetate of the normal 20-keto compound with 0.5 N NaOH in 80 per cent ethanol for 1 hour. The 17α-pregnane derivative was more soluble in ethyl acetate than the normal epimer and about 40 per cent of pure 17β isomer was obtained from the mixture by fractional crystallization. The mother liquors were difficult to separate, but in one instance it was possible to obtain from ethyl acetate a crop of nearly pure 3α,11α-dihydroxy-17α-pregnan-20-one as fine silky needles melting in the range 110–120°. Chromatography on alumina enriched the early fractions in the 17α derivative, but the product was still a mixture. High vacuum sublimation at 110–115° yielded a glassy sublimate which crystallized from ethyl acetate and consisted largely of the 17α epimer with a small amount of the normal compound. Continuation of the sublimation at 125–135° yielded a product which contained the normal isomer together with a small amount of lower melting needles of the 17α compound. Proof that the lower melting needles were an isomerization product and not an unidentified contaminant in the original diacetate was obtained by heating 99 mg. of nearly pure 3α,11α-dihydroxy-17β-pregnan-20-one ($[\alpha]_D$ = +93° in chloroform;

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pure substance $[\alpha]_D = +96°$) under a reflux with alcoholic base. After collecting four crops of the normal isomer, 14 mg. of fine silky needles, identical with the lower melting levorotatory isomer, were obtained. Had this minimal amount of 17α derivative been initially present as a contaminant, the rotation of the original product could have been no higher than +73°. Further proof that the lower melting needles were a 17α compound was obtained when practically all of the isomerization product was converted to the normal isomer after six successive reisomerizations.

When essentially pure 3α,11α-dihydroxy-17β-pregnan-20-one was converted to 3α,11α-diacetoxy-21-benzalpregnan-20-one, isomerization at C-17 again occurred. This unwanted rearrangement very probably took place during the acetylation of 3α,11α-dihydroxy-21-benzal-17β-pregnan-20-one, although this has not been rigidly proved, since the formation of the benzal was effected with sodium ethylate at −3°. Acetylation of 3α,11α-dihydroxy-21-benzalpregnan-20-one (m.p. 215–219°; pure compound 219–220°) was achieved by heating under a reflux for 1 hour with acetic anhydride and pyridine. Five crystalline forms described in Table I were isolated, three of which were known to be polymorphic modifications of the normal diacetate. The two remaining products had the same rotation, differing from the normal by approximately 110° and were thus undoubtedly polymorphic forms of the 17α epimer. Both levorotatory products exhibited the characteristic ultraviolet absorption spectrum of a 20-ketobenzal (1) (ϵ_{2940} = 23,400; ϵ_{2940} = 22,900) and were therefore not enol acetates, since these have a much higher molecular extinction coefficient with maxima at 2930 A and 3030 A (5). These were separated from a small sample only. The principal quantity of the mixture of five crystal-

Table I

Crystalline Modifications of 17α and 17β Epimers of 3α,11α-Diacetoxy-21-benzalpregnan-20-one

Configuration C-17	Crystal form	M.p.	ϵ_{2940} (ethanol)	$[\alpha]_D$ ($CHCl_3$)
		°C.		*degrees*
β	Needles	162–163	22,500	+71
"	" *	116–120	24,000	+70
"	Prisms	162–163		+70
α	Spikes	166–168	23,400	−37
"	Rods	180–182	22,900	−41

* This form was obtained when the compound was dried at a temperature below 80°. Upon drying at 100° and 0.1 mm. for 1 hour, the product was converted to needles melting at 162–163°.

line forms was degraded to the etio acid by ozonolysis and periodic acid oxidation. Two isomeric etio acids were obtained from the reactions, the 17α in about 30 per cent yield. Confirmation of the structure of the 17α-etio acid was obtained by esterifying 640 mg. with diazomethane followed by isomerization with sodium methoxide in methanol by the procedure of Sorkin and Reichstein (2). 376 mg. (61 per cent) of the higher melting normal acid and 72 mg. (11.6 per cent) of the 17α acid were isolated after saponification.

It is clear from these results and from those previously reported (5) that enolization of a 20-ketosteroid, especially the highly conjugated 20-keto-21-benzalpregnane derivative, is a facile reaction despite the formation of an exocyclic double bond. Acetylation of hydroxyl groups in these steroids can be more readily and safely accomplished in the cold by the perchloric acid-catalyzed reaction of Whitman and Schwenk (6) rather than by prolonged heating in pyridine. It should also be noted, as previously reported (1), that saponification of 3α,11α-diacetoxypregnan-20-one can be effected at room temperature for 2 days in a solution of 2 N NaOH in 75 per cent ethanol without appreciable isomerization.

Table II summarizes the difference in molecular rotation recorded for pairs of 20-ketosteroids and derivatives epimeric at C-17. It is apparent that the acetyl group attached to C-17 in the α configuration causes a pronounced levorotatory shift so that the difference in molecular rotation between the two configurations of C-17 is approximately 55,000. This value can be a criterion of purity or completeness of separation of the isomers and the usefulness of this value is illustrated by the following considerations. Butenandt and Mamoli (13) have described 3β-hydroxy-17α-allopregnan-20-one with $[\alpha]_D = +6°$ (ethanol). The difference in molecular rotation between this product and the 17β isomer ($[\alpha]_D = +90.8°$ in ethanol (14)) is 27,000, from which the conclusion can be drawn that only a 50 per cent separation was achieved; it is possible that the product isolated was a 1:1 molecular compound of the 17α and 17β isomers. Experimental confirmation of the inhomogeneity of the 17α-20-ketone isolated by Butenandt and Mamoli is found in the same report of these authors who isolated both 17α- and 17β-allopregnane-3,20-dione from CrO_3 oxidation of 3β-hydroxy-17α-allopregnan-20-one. It is probable that the 17α-allopregnane-3,20-dione isolated from this reaction still contained an appreciable amount of the 17β epimer, since the $\Delta[M]_D$ is the smallest of all the reported diastereoisomeric pairs. For these reasons the 17α and 17β isomers of 3β-hydroxy-allopregnan-20-one were not included in Table II and the value for 17α-allopregnane-3,20-dione should be interpreted in the light of the foregoing discussion. From the value $\Delta[M]_D^{17\alpha-17\beta} = 55{,}000$ it can be concluded that the hydroxyl group at C-11 does not exert any pronounced vicinal effect

TABLE II

Molecular Rotations of 20-Ketosteroids Epimeric at C-17

Isomeric pair No.	Derivatives of 20-ketopregnane (or pregnene)	$[\alpha]_D$	Solvent	Bibliographic reference No.	$[M]_D$	$[M]_D^{17\beta}$- $[M]_D^{17\alpha}$
		degrees				
1	3α-Hydroxy-17β	+112	MeOH	(7)	36,300	51,400
	3α-Hydroxy-17α	−49.7	EtOH	*	−15,100	
	3α-Acetoxy-17β	+123	"	(8)	44,700	55,700
	3α-Acetoxy-17α	−28.2	MeOH	(7)	−11,000	
		−30.6	EtOH	*		
2	Δ^5-3β-Hydroxy-17β	+28.2	"	(9)	8,900	53,400
	Δ^5-3β-Hydroxy-17α	−140.5	"	(9)	−44,500	
	Δ^5-3β-Acetoxy-17β	+19.9	"	(9)	7,200	52,400
	Δ^5-3β-Acetoxy-17α	−126	"	(9)	−45,200	
3	Δ^4-3,20-Dione-17β	+187	"	(10)	58,800	58,800
	Δ^4-3,20-Dione-17α	0	"	(10)	0	
4	Δ^4-3-Keto-21-hydroxy-17β	+178	"	(11)	58,800	60,800
	Δ^4-3-Keto-21-hydroxy-17α	−6	"	(12)	−2,000	
	Δ^4-3-Keto-21-acetoxy-17β	+170	Acetone	(11)	63,300	
	Δ^4-3-Keto-21-acetoxy-17α	−21	"	(12)	−7,800	71,100
		−26	"		−9,700	73,000
5	3,20-Dione-5α,17β	+127	EtOH	(13)	40,200	44,800
	3,20-Dione-5α,17α	−14.6	"	(13)	−4,600	
6	3α,11α-Dihydroxy-17β	+96	$CHCl_3$		32,100	55,200
	3α,11α-Dihydroxy-17α	−69	"		−23,100	
	3α,11α-Diacetoxy-21-benzal-17β	+71	"		36,000	56,800
	3α,11α-Diacetoxy-21-benzal-17α	−41	"		−20,800	

* The product was obtained from an equilibrium mixture of 17α and 17β pregnanolone generously furnished us by Dr. Willard Hoehn. Careful chromatographic separation yielded 3α-hydroxy-17α-pregnan-20-one which melted from 137–144° (Kofler block); $[\alpha]_D = -50°$ (ethanol or dioxane); the properties of this product were not changed by rechromatography or by fractional crystallization. The unsharp melting point is believed to be due to the presence of two crystal modifications. The acetate melted at 158.5–160°; $[\alpha]_D^{27} = -30.6°$ (ethanol). Comparison of the infra-red spectrum of the hydroxyketone with an authentic sample obtained from Moffett and Hoehn failed to reveal any difference between the two products. Dr. K. Dobriner and Dr. J. Hardy of the Sloan-Kettering Institute, who examined the infra-red spectra, inform us that, while the spectra of 3α-hydroxy-17β-pregnan-20-one and 3α-hydroxy-17α-pregnan-20-one are not identical, the fact that the strong bands of the two compounds overlap to a great extent makes the detection of small amounts of the 17α epimer in the 17β compound extremely difficult. From the rotation data Moffett and Hoehn's product still contained approximately 8 per cent of the 17β isomer. We wish to express our thanks to Dr. Hardy, Dr. Dobriner, and Dr. Hoehn for their assistance.

upon the rotatory contribution of C-17. In contrast the vicinal effect of a C-21 hydroxyl (Pair 4), while slight, is apparent and is increased by acetylation; the vicinal effect of the 21-benzal group appears negligible (Pair 6).

A similar treatment of the etio acids and their simple derivatives, summarized in Table III, shows that the carboxyl group or methyl carboxylate attached in the α or β configuration to C-17 makes a considerably smaller contribution to the molecular rotation than the 17 acetyl group. The assignment of a numerical value to the $\Delta[M]_D^{17\alpha-17\beta}$ is complicated by the greater disparity in the value for these compounds. This may in part be due to the unfortunate fact that the rotation was determined in a different solvent for the members of the epimeric pair. The two pairs which differ most markedly from the other compounds are substances with hydroxyl functions at C-11 or C-12. Here at least three explanations are plausible: (1) that the separation of the products was incomplete; (2) that there is vicinal effect of the hydroxyl function at either C-12 or C-11; or (3) that the relatively small rotations were not determined with sufficient accuracy. It is significant that the $\Delta[M]_D^{17\alpha-17\beta}$ for the 17 epimers of methyl 3α, 11α-diacetoxyetiocholanate is the largest observed in the series. Unfortunately it is not possible to decide which of the three possibilities mentioned is responsible for this result and further investigation is required in order to clarify these inconsistencies.

TABLE III

Molecular Rotations of Etiocholanic Acids and Derivatives Epimeric at C-17

Isomeric pair No.	Derivatives of etiocholanic acid (or etiocholenic)	$\alpha]_D$	Solvent	Bibliographic reference No.	$[M]_D$	$[M]_D^{17\beta}$- $[M]_D^{17\alpha}$
		degrees				
1	Methyl-5α, 17β	+49	Dioxane	(15)	15,600	28,600
	Methyl-5α, 17α	+41	"	(15)	−13,000	
2	Methyl-3β-acetoxy-5α, 17β	+37	$CHCl_3$	(2)	16,100	32,200
	Methyl-3β-acetoxy-5α, 17α	−37	"	(2)	−16,100	
3	Methyl-3β-acetoxy-5β, 17β	+54	Acetone	(2)	20,300	30,800
	Methyl-3β-acetoxy-5β, 17α	−28	$CHCl_3$	(2)	−10,500	
	Methyl-3β-hydroxy-5β, 17β	+57	Acetone	(2)	19,100	29,500
	Methyl-3β-hydroxy-5β, 17α	−31	$CHCl_3$	(2)	−10,400	
4	Methyl-3α, 12α-dihydroxy-17β	+105	MeOH	(16)	36,800	33,200
	Methyl-3α, 12α-dihydroxy-17α	+10	$CHCl_3$	(16)	3,600	
5	Methyl-3α, 12β-dihydroxy-17β	+52	MeOH	(16)	18,200	21,400
	Methyl-3α, 12β-dihydroxy-17α	−9	$CHCl_3$	(16)	−3,200	
6	Methyl-Δ⁴-3-keto-17β	+145	Acetone	(15)	47,900	37,000
	Methyl-Δ⁴-3-keto-17α	+36	"	(12)	10,900	
7	3α, 11α-Dihydroxy-17β	+60	EtOH		20,200	23,600
	3α, 11α-Dihydroxy-17α	−10	"		−3,400	
	Methyl-3α, 11α-diacetoxy-17β	+46	$CHCl_3$	(1)	20,000	41,700
	Methyl-3α, 11α-diacetoxy-17α	−50	"		−21,700	

We have attempted to establish the equilibrium of the normal and iso-20-

ketopregnanes by measurement of the shift in the optical rotation upon heating for 30 minutes at 80° in 0.5 N NaOH. From the results presented in Table IV it would appear that 66 per cent of the 17β compound and 34 per cent of the 17α represent the equilibrium condition. These results should be compared with those of Moffett and Hoehn (7), who carried out similar experiments with 3α-hydroxypregnan-20-one and reported that the equilibrium mixture contained 71 per cent of the 17β compound and 29 per cent of the 17α. Recalculation of their results with our value $[\alpha]_D = -50°$ for the 17α-20-ketosteroid shows the equilibrium mixture to contain approximately 75 per cent of the 17β and 25 per cent of the 17α compound.

TABLE IV

Rotatory Shift of Epimers of 3α,11α-Dihydroxypregnan-20-one

Epimer	Specific rotation at 23°		
	80 per cent EtOH alone	0.5 N EtOH-NaOH unheated	0.5 N EtOH-NaOH heated 30 min., 80°
	degrees	*degrees*	*degrees*
17β	+89	+92	+48
17α	−50	−50	+40

EXPERIMENTAL[1]

3α,11α-Dihydroxy-17α-pregnan-20-one—7.78 gm. of 3α,11α-diacetoxy-17β-pregnan-20-one, melting in the range 145.5–147°, were heated under a reflux for 1 hour in 120 ml. of 80 per cent ethanolic 0.5 N NaOH. Crystallization from ethyl acetate yielded 2.80 gm. of platelets of the normal isomer, but additional crops were mixtures of platelets and needles. Mother liquors and crystals melting below 170° were combined and a series of six isomerizations (heating in ethanolic base as above) alternated with fractional crystallization from ethyl acetate yielded 4.97 gm. (80 per cent) of pure 3α,11α-dihydroxypregnan-20-one together with 60 mg. of less pure product, 660 mg. of crude mixed isomers, and 187 mg. of needles melting in the range 110–120°. The calculated yield was 6.2 gm. and 5.8 gm. were realized. The 17α compound was recrystallized from ethyl acetate-petroleum ether as fine silky needles and after repeated purification yielded a product which melted at 120–123° with bubbles; $[\alpha]_D^{23} = -69°$ (chloroform); $[\alpha]_D^{23} = -50°$ (80 per cent ethanol). The behavior on melting appeared to be due to solvation, but the analytical specimen dried at 80° and 0.1 mm. for 4 hours showed no loss in weight.

$C_{21}H_{34}O_3$. Calculated, C 75.41, H 10.25; found, C 74.04, H 10.65

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[1] All melting points are corrected.

A second analytical specimen, 20 mg. of needles melting at 118–120°, was sublimed in high vacuum at 110–115°. The analysis of the transparent glassy sublimate, although it exhibited a specific rotation identical with that of the crystalline needles, $[\alpha]_D^{23} = -69°$ (chloroform), was similarly poor. Found, C 74.28, H 12.70.

99 mg. of 3α,11α-dihydroxy-17β-pregnan-20-one, m.p. 179–180.5°, $[\alpha]_D^{23} = +93°$ (chloroform), were heated under a reflux in an atmosphere of nitrogen for 1 hour in 5 ml. of 80 per cent ethanolic 0.8 N NaOH. The solution was cooled, neutralized, diluted with 100 ml. of water, and extracted three times with ether. The combined extracts were washed with dilute brine, dried over sodium sulfate, and the solvent removed under diminished pressure. The dried residue yielded four crops of the normal isomer from ethyl acetate, which melted in the range 150–174°. The next two crops were needles, weighing 14 mg. and melting at 110–115°; $[\alpha]_D^{24} = -57°$ (chloroform).

Rotatory Shift of 17β and 17α Epimers upon Heating in Alcoholic Base—10.9 mg. of platelets melting at 179–180.5° were dissolved in 4 ml. of 80 per cent ethanolic 0.5 N NaOH. 10.6 mg. of needles melting at 118–120° were similarly treated. The solutions were prepared under nitrogen and the rotation was determined within 15 minutes. Each was then transferred to a bomb tube under nitrogen, and the tubes sealed and heated for 30 minutes at 80°. After cooling, the rotation of each solution was again determined. The results are shown in Table IV.

3α,11α-Dihydroxy-17α-etiocholanic Acid—4.46 gm. of the mixture of 17α and 17β isomers of 3α,11α-diacetoxy-21-benzalpregnan-20-one were subjected to ozonolysis and periodic acid fission by the procedure previously reported (1). The crude acidic fraction weighed 2.62 gm. (calculated, 2.96 gm.), but, upon crystallization from ethyl acetate, the third crop melted at 200–204°. Upon recrystallization, this product yielded a small amount of needles melting at 240° of the 17β acid and 915 mg. of the 17α acid in the form of prisms. Recrystallization from ethyl acetate or dilute ethanol gave micro prisms melting at 204.5–205.5°; $[\alpha]_D^{24} = -10°$ (95 per cent ethanol).

$C_{20}H_{32}O_4$. Calculated, C 71.39, H 9.59; found, C 71.41, H 9.65

A mixture with the 17β isomer, m.p. 250°, showed no depression of the melting point of the lower melting component and melted at 200–206°.

Conversion of 3α,11α-Dihydroxy-17α-etiocholanic Acid to Normal Isomer—644 mg. of 3α,11α-dihydroxy-17α-etiocholanic acid, melting within the range 199–205°, were esterified with diazomethane and the crude ester dried by distilling off two 25 ml. portions of anhydrous benzene, leaving 2 ml. of benzene on the residue. 25 ml. of a 5 M solution of sodium methoxide in methanol were quickly added, the flask connected to a condenser, and the mixture boiled under a reflux in an atmosphere of nitrogen. After 30 minutes boiling, 15 ml. of water were added and boiling continued for an additional 15 minutes. The solution was diluted with 50 ml. of water and methanol was removed under reduced pressure. Upon cooling the solution, the insoluble sodium salt of the 17α acid precipitated. The insoluble salt was collected on a fritted disk and washed with a little water. The filtrate was chilled, diluted with water, acidified to Congo red with 10 per cent sulfuric acid, and sodium chloride was added to a concentration of 10 per cent. The suspension was extracted with ether and the ether extracts were washed with dilute brine, dried over sodium sulfate, and the solvent removed. The residue weighed 449 mg. and yielded 376 mg. of the normal isomer melting in the range 240–248°. The insoluble sodium salt was converted to the free acid and isolated in similar fashion. 184 mg. of the crude acid were obtained from which 72 mg. of crystalline 17α-etio acid, identical in all respects with the product described, were obtained. The mother liquors were not investigated, but were combined with other mixtures of the isomers for reisomerization.

Methyl 3α,11α-diacetoxy-17α-etiocholanate was isolated from a sample of the mixture of isomers which had been esterified with diazomethane and acetylated with acetic anhydride in the presence of $HClO_4$. It was separated from the 17β isomer by chromatography upon Al_2O_3, from which it was eluted before the 17β epimer. It crystallized from petroleum ether as needles, m.p. 141–142°, $[\alpha]_D^{24} = -50°$ ($CHCl_3$).

$C_{25}H_{38}O_6$. Calculated, C 69.10, H 8.81; found, C 69.13, H 8.74

SUMMARY

Two new steroids and their derivatives with the 17α or unnatural configuration of the side chain have been described. The molecular rotation differences of the 17α and 17β epimers have been compared with other pairs of similar epimers.

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THE EGG WHITE INHIBITOR OF INFLUENZA VIRUS HEMAGGLUTINATION

I. PREPARATION AND PROPERTIES OF SEMIPURIFIED INHIBITOR*

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PLATE 4

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Several fluids of biological origin, notably, normal serum, normal allantoic fluid, ovarian cyst fluid, and egg white, have recently been shown to inhibit markedly the agglutinative reaction between red blood cells and heated influenza viruses, and the results of studies on the purification of inhibitor from each of these fluids have already been reported (1–4). Of these materials, egg white, which is the most readily available, appears possibly to be one of the richest sources of inhibitor (5). Accordingly, experiments have been undertaken in this laboratory for the purpose of purifying and characterizing the inhibiting component of egg white. The products have been investigated with respect to biological activity, chemical composition, appearance in electron micrographs, sedimentation and electrophoretic behavior, and other properties.

Materials and Methods

The *swine influenza virus* for titrating the inhibitor was the same preparation of egg-adapted strain 15 cultured in chick embryos and concentrated by Sharples centrifugation of virus-infected chorioallantoic fluid previously described (6). The concentrate was passed through one cycle of alternate low and high speed spinning in the vacuum type ultracentrifuge. The nitrogen content of the final suspension was 234 γ per ml. The 50 per cent point infectious unit of this virus preparation was $10^{-14.7}$ gm. of N on February 9, 1948, 3 months after concentration and purification. Titration on December 6, 1948, revealed a 50 per cent point unit of $10^{-12.9}$ gm. of N, a decline of about two 10-fold dilutions in infectious capacity.

Inhibitory activity was titrated against a constant amount of heated

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† Predoctorate Fellow of the United States Public Health Service, National Cancer Institute.

virus, prepared by diluting the stock virus described above to 1 γ of N per ml. with *buffered saline* (0.81 per cent NaCl, 0.005 M phosphate, pH 7.3) and heating for 30 minutes at 53°.[1] Progressive 2-fold dilutions of inhibitor solutions were made in bulk with buffered saline, and 0.5 ml. portions were distributed in Wassermann tubes. A volume of 0.5 ml. of heated virus, diluted to contain about eight hemagglutinating doses per ml., was added, and the mixtures were allowed to stand for 30 minutes at room temperature. After this time, 1.0 ml. of 2 per cent chicken red blood cells was added, and the agglutination readings, recorded as 0 to ++++, were made as usual (8) after 1 hour. The *inhibition titer* of a preparation is defined as the reciprocal of the final dilution of the preparation in the reaction mixture which gave the standard ++ end-point of hemagglutination; ++ hemagglutination is given by one *hemagglutinating dose* of virus. Most of the end-points were obtained by interpolation from the results with two adjacent mixtures, the first giving less and the second giving more than the standard end-point agglutination. Duplicate determinations generally agreed to within 10 per cent. The *purification factor* of a fraction, defined as the ratio of egg white N to fraction N at the ++ end-point, was calculated from the results of parallel titrations. In the present experiments, the character of the hemagglutination curve in the gradient region was independent of the purity of the inhibitor-containing solutions. This independence was observed with materials, including crude egg white, highly purified fractions, and residues, which differed as much as 300 times in purification factor. Furthermore, a mixture of a purified fraction with purification factor 34 and a residue with a factor of 0.15 had an inhibitory activity equal (within 10 per cent) to the sum of the activities of the separate fractions; the two fractions were allowed to contribute approximately the same activity to the mixture. These two features of the titrations indicate that the inhibition titer is independent of purity (*cf.* (2)).

Sedimentation studies were made with the air-driven analytical ultracentrifuge (9) by the schlieren or Lamm scale methods. The *electron microscope* was the RCA type B instrument (10). *Electrophoretic* studies, by means of the Tiselius electrophoresis apparatus equipped with a Svensson optical system (11), were made both in veronal and in phosphate buffers of 0.1 ionic strength. *Ultraviolet absorption* studies were made with the Beckman quartz spectrophotometer. The *viscosity* determinations were carried out in capillary viscometers (12) at 29.78°. *Nitrogen* was determined either by a micro-Kjeldahl distillation method or by a direct nesslerization method adapted to the determination of quantities from 5 to 80 γ. *Carbohydrate* was determined with the orcinol reagent (13) and calculated as glucose.

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[1] Unheated virus is not suitable for the titrations because it inactivates inhibitor rapidly (6). Heated virus, prepared as described, is essentially devoid of inhibitor-inactivating capacity (7).

Results

Fractionation—1 volume (200 ml.) of fresh egg white, strained through fine gauze, was poured into 7 volumes of 0.1 M KH_2PO_4, and the mixture (pH 5.7 by the glass electrode) was allowed to stand 1 hour at room temperature with frequent swirling. The stringy precipitate was obtained by centrifuging (supernatant fluid = SI), washed once with 2 volumes (referred to egg white) of 0.085 M phosphate buffer at pH 5.7, and suspended in 1 volume of 0.06 M phosphate buffer at pH 7.2. A stage sample of 1.0 ml. of the suspension was dispersed in 9.0 ml. of phosphate buffer at pH 7.2 and centrifuged, giving the supernatant fluid PI for analysis. The remainder of the suspension was precipitated twice with 5 volumes of 0.088 M KH_2PO_4 each time, giving the supernatant fluids SII and SIII, corresponding to SI, and the stage samples PII and PIII, corresponding to PI. The final highly viscous suspension, in unit volume referred to egg white, was shaken briefly and centrifuged, giving the extract PIII-EI. The undissolved, gelatinous material was then extracted twice more with 0.5 volume of phosphate buffer at pH 7.2 each time, giving the fractions PIII-EII and PIII-EIII. At each step in the foregoing procedure, the material was allowed to stand for at least 1 hour.

TABLE I

Properties of Egg White Fractions Obtained with Phosphate Buffers (Experiment A178)

Fraction (see text)	Volume (unit volume 200 ml.)	Inhibition titer	N	Purification factor	Distribution of activity
			γ per ml.		per cent
Egg white	1	47,000	18,200	1.00	100
SI	8	1,100	2,170	0.19	19
Wash	2	320	85.4	1.4	1.4
PI*	10	470	(2.5)†	(72)	10‡
SII	6	1,100	11.4	37	14
PII	10	1,800	19.9	35	38‡
SIII	6	350	(3.0)†	(45)	4.5
PIII	10	1,400	13.0	41	30‡
PIII-EI	1	16,000	148	41	34
PIII-EII	0.5	7,500	46.5	62	8.0
PIII-EIII	0.5	930	5.7	62	1.0

* Inadequately dispersed.

† Nitrogen too low for accurate analysis.

‡ The data apply to stage samples and are not to be included in the sum.

The results of Table I show that approximately 80 per cent of the egg white inhibitory activity was precipitated with approximately 5 per cent of the egg white nitrogen in the initial step. Washings after the first (other experiments) and subsequent precipitations contributed little to the purification. On the other hand, the first extract (*e.g.*, PIII-EI) of a given precipitate was not as pure as subsequent extracts, possibly because the removal of inert material was facilitated by the swelling which occurred when the precipitate, always difficult to disperse and dissolve, was treated with phosphate buffer at pH 7.2.

Several alternative methods of purification have been investigated briefly: (*a*) dilution of egg white with several volumes of water; (*b*) dilution with 7 volumes of 0.1 M acetate buffers; (*c*) half saturation with ammonium sulfate; and (*d*) fractionation by the method of Sørensen (14). In each case the activity was concentrated in the least soluble fraction, but none of these methods, which were explored only in a preliminary way, gave products more than 10 times as active as egg white on an N basis.

When it was found that the egg white inhibitor could be sedimented in high centrifugal fields (*cf.* (4)), several purification experiments were carried out, utilizing this property of the inhibitor. Egg white, diluted 5-fold with 0.06 M phosphate buffer at pH 7.2, was centrifuged for 90 minutes at 67,000 × *g* in the vacuum type quantity ultracentrifuge. The supernatant fluid (UC-SI) was pipetted off, and the gelatinous, runny pellets were washed with 0.085 M phosphate buffer at pH 5.7, pooled, and extracted with 0.06 M phosphate buffer at pH 7.2. The extract (UC-PI) was clarified at low speed and centrifuged for 60 minutes at 67,000 × *g*, giving a supernatant fluid (UC-SII) and gelatinous pellets, which were extracted with buffer at pH 7.2 (UC-PII). The cycle was repeated, giving the supernatant fluid UC-SIII and the pellet extract UC-PIII. The properties of these fractions are summarized in Table II. While this method gave products comparable in purity with those obtained by the phosphate buffer method, the latter is superior, since it allows the handling of a greater volume of material.

Recently, Hardy and Horsfall (4) reported that they were able to obtain by cold alcohol precipitation of egg white a fraction which was approximately 5.4 times[2] as active as egg white on the basis of dry weight, as tested against the PR8 strain of influenza virus A. The most purified preparations obtained in the present work, *e.g.*, Preparation A178-PIII-EII of Table I, were approximately 60 times as active as egg white on a nitrogen basis and approximately 55 times as active on the basis of dry weight, assuming a nitrogen content of 12.5 per cent (see below).

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[2] Calculated from their report that 0.25 per cent egg white and a solution of purified material containing 0.06 mg. per ml. each had the same inhibitory activity as normal allantoic fluid. Egg white contains 13.0 per cent total solids (15).

General Properties—The inhibitor solutions contained up to 300 γ of N per ml. in 0.06 M phosphate buffer at pH 7.2. In general, the purification factor of these preparations was inversely related to the nitrogen concentration; no preparations with purification factor 60 were obtained in concentrations greater than 60 γ of N per ml. The preparations were characterized by a blue scattering, a high viscosity (see below), and a tendency to form shreds of denatured material on swirling or on shaking with air. One preparation, A187-PI-EI (Table III), was shaken with air for 1 hour at room temperature (26°) and centrifuged at low speed to remove the considerable precipitate which formed. The supernatant fluid contained 3 per cent of the inhibitory activity and 13 per cent of the nitrogen of the starting material. Preliminary experiments showed that the thoroughly washed sediment was capable of combining with both heated and unheated swine influenza virus. The same preparation was heated at 90° for 1 hour with no change in activity (*cf.* (1, 2, 4, 16, 17)). A second preparation, A178-PIII-EI (Table I), was dialyzed in Visking casing against buffered saline (pH 7.3) for 48 hours in the cold with a decrease of 6 per cent in inhibitory activity and 10 per cent in nitrogen concentration. No optical activity or birefringence of flow could be demonstrated.

TABLE II

Properties of Egg White Fractions Obtained with Ultracentrifuge (Experiment A177)

Fraction (see text)	Volume (unit volume 207 ml.)	Inhibition titer	N	Purification factor	Distribution of activity
			γ per ml.		*per cent*
1:5 egg white.....	1.0	9,600	3500	1.00	100
UC-SI............	1.0	2,000	3200	0.23	21
Wash.............	0.33	3,200	366	3.2	11
UC-PI............	0.27	12,000	202	22	34*
UC-SII...........	0.27	4,100	139	11	11
UC-PII...........	0.21	5,400	53.9	37	12*
UC-SIII..........	0.21	1,600	17.5	33	3.5
UC-PIII..........	0.14	1,200	9.0	49	1.8

* The data apply to stage samples and are not to be included in the sum.

Chemical Properties—A preparation containing 60 γ of N per ml., with purification factor 60, gave positive Millon's, xanthoproteic, and biuret tests and a doubtful ninhydrin test for protein. A positive test for carbohydrate was obtained with the orcinol reagent, but the Molisch test was negative (*cf.* (4)).

Preparation A178-PIII-EI (Table I) was dialyzed exhaustively against flowing distilled water,[3] frozen, and dried *in vacuo*, yielding a white, cohesive, flexible shell, which did not readily redissolve in 0.06 M phosphate buffer at pH 7.2. The yield of lyophilized material was 1.25 mg. per ml. of starting material. After further drying to constant weight *in vacuo* over P_2O_5 at 50°, analyses gave for C 48.1, H 6.7, N 12.5, S 2.4, and ash 1.4 per cent; no phosphorus was present.[4] The carbohydrate content, determined with the orcinol reagent on the dry material and calculated as glucose, was 10.3 per cent.

Viscosity and Carbohydrate Content in Relation to Inhibitory Activity—Fractions obtained by the phosphate buffer method described above, with slight modifications, were analyzed for N, carbohydrate (orcinol), viscosity, and inhibitory activity, with the results shown in Table III. The recorded values refer to solutions at the concentration at which the viscosity measurements were made. It is evident that progressive purification of the active component is accompanied by progressive increase in the viscosity:N and carbohydrate:N ratios; plots of each of these ratios against the titer:N ratio show an essentially linear relation. It would appear that the inhibitory activity is associated with a highly viscous

TABLE III

Viscosity and Carbohydrate Content of Egg White Fractions in Relation to Inhibitory Activity (Experiment A187)

Fraction*	N	Inhibition titer	Carbohydrate (orcinol)	Specific viscosity†	$\frac{\text{Titer}}{\text{N}}$	$\frac{\text{Carbohydrate}}{\text{N}}$	$\frac{\text{Specific viscosity} \times 10^3}{\text{N}}$
	γ *per ml.*		γ *per ml.*				
10% egg white	1700	4,800	928	0.1304	2.8	0.55	0.0768
15% SI	299	130	150	0.0088	0.43	0.50	0.029
PI-EI	290	18,000	262	0.615	62	0.90	2.12
PI-EII	238	24,000	250	0.945	100	1.05	3.97
PI-EIII	183	23,000	224	0.819	130	1.22	4.48
10% thin egg white	1760	1,600		0.0824	0.91		0.0468
10% thick " "	1750	8,800		0.1552	5.0		0.0887

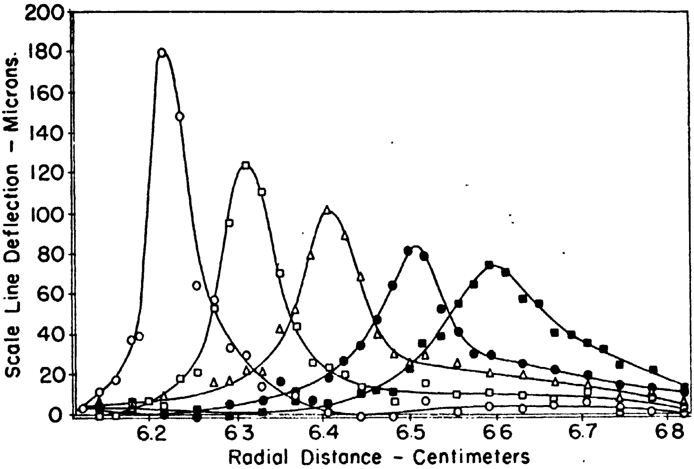
* All the fractions were in 0.06 M phosphate buffer at pH 7.2, containing 0.01 per cent merthiolate (Lilly).

† Relative viscosity minus 1.

[3] Very little precipitate formed during this dialysis, which was carried out on a rocking device in the cold without the inclusion of an air bubble. In other experiments, variable amounts of precipitate formed when air was included.

[4] These analyses were made by the Oakwold Laboratories, V. A. Conard, Director, Alexandria, Virginia.

component which has a carbohydrate content, as determined with orcinol, appreciably greater than the average egg white content. Furthermore, the data suggest that the inhibitor itself, contributing 2 per cent or less of the total egg white N, is responsible for a great part of the high viscosity of crude egg white. This conclusion is supported by a comparison of the properties of thin and thick whites separated from the same eggs (Table III). The probable identity of inhibitor and viscous component is affirmed by the recent observation (18) that purified swine influenza virus is capable of reducing greatly the viscosity of solutions of purified inhibitor; the inactivation of inhibitor by virus has already been reported (6, 7).



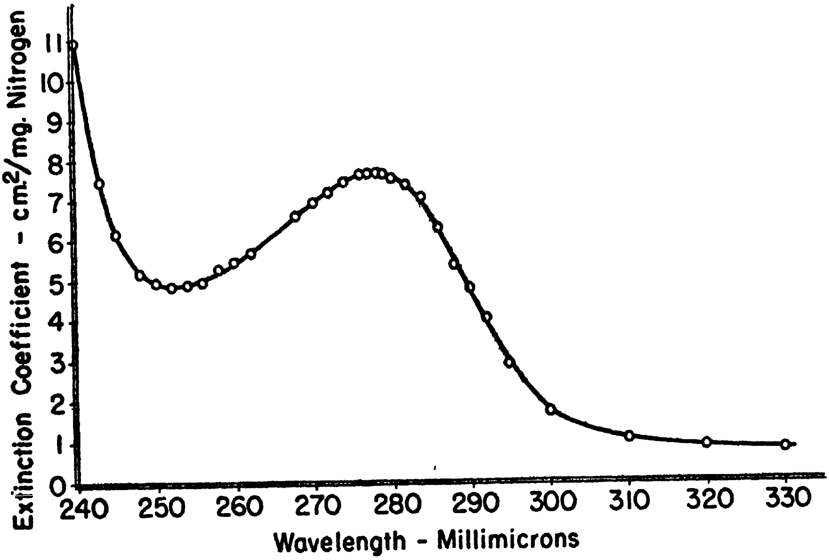
TEXT-FIG. 1. Sedimentation diagram of a preparation of the egg white inhibitor (Preparation A187-PI-EIII, Table III). The boundary, sedimenting in a mean centrifugal field of 46,450 × g, is shown in five positions by the five scale curves taken at intervals of 10 minutes. The preparation contained 183 γ of N per ml. The sedimentation constant was 31 S.

Sedimentation Behavior—Sedimentation studies were made with several preparations containing from 75 to 183 γ of N per ml. in 0.06 M phosphate buffer at pH 7.2. At 75 γ of N per ml. a boundary was barely visible, but at concentrations above this level, distinct boundaries were seen. A Lamm scale diagram of Preparation A187-PI-EIII (Table III), containing 183 γ of N per ml., with purification factor about 45, is shown in Text-fig. 1. The diagram shows a single, fairly sharp boundary, spreading somewhat in the course of sedimentation. The spread of the boundary is comparable with that of the boundaries of the influenza viruses (19).

The sedimentation constants obtained with various preparations ranged from 31 to 37 S. The sedimentation constant of the preparation of Fig. 1 was 31 S. As with other highly viscous materials (20), a definite relation was seen between the rate of sedimentation and the concentration of the inhibitor, the rate being slower at the higher concentration.

Calculations of the particle size of the sedimenting material on the assumption of a spherical shape and a hydrated density of 1.25 give a value of about 15 mμ for the mean diameter.

Electron Micrography—The inhibitor preparations in 0.06 M phosphate buffer at pH 7.2 were diluted 1:10 to 1:100 with distilled water. Collodion films were prepared in the usual way, and the material dried on the film was lightly shadow-cast with chromium. A micrograph (Fig. 1) of preparation UC-PI (Table II) diluted 1:10 with water shows images of widely varying size closely crowded together. Most of the individual particles are exceedingly small and approximately spherical, while the larger images have the appearance of aggregates of the smaller particles. A micrograph (Fig. 2) of preparation A178-PIII-EI (Table I) diluted 1:100 reveals particles in the same size range as the individual small particles visible in Fig. 1. Neither micrograph shows unequivocal evidence of systematic aggregation of the particles in all parts of the field, although there is some suggestion of a thread-like aggregation in localized areas of Fig. 2. It is doubtful whether this appearance is wholly an artifact,



TEXT-FIG. 2. Ultraviolet absorption spectrum of a preparation (A178-PIII-EI, Table 1) of egg white inhibitor.

since the orientation of the threads is independent of the direction of shadows, though it is possible that the thread appearance was produced in the drying process. Calculations from measurements of the images give a diameter of about 10 to 20 mμ for the particles of predominant size.

Ultraviolet Absorption—The ultraviolet absorption spectrum of Preparation A178-PIII-EI (Text-fig. 2) shows a maximum at 2780 A, in the region typical of most proteins. The dependence of optical density on concentration was essentially linear, as determined at the maximum.

Electrophoresis—Examination of Preparation A178-PIII-EI (Table I) in veronal buffer of pH 8.6 and ionic strength 0.1 showed two well defined components, one, with mobility -3.5×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, comprising 71 per cent, and the other, with mobility -7.17×10^{-5}, comprising 29 per cent of the total electrophoretic area. In a further experiment, 15 ml. of the same preparation were centrifuged twice for 20 minutes at 40,000 × *g* each time, the supernatant fluid being collected with a pipette. Tests of the final supernatant fluid showed that approximately 28 per cent of the initial nitrogen, but very little of the inhibitory activity, had been sedimented. Electrophoresis in phosphate buffer of pH 7.2 and ionic strength 0.1 showed again two components with mobilities of -3.05 and -7.54, comprising 70 and 30 per cent, respectively, of the total area.

DISCUSSION

Identity of Inhibitor—Various properties of the preparations obtained here indicate that the egg white inhibitor of influenza virus hemagglutination is a carbohydrate-containing protein of high molecular weight. While the data are not yet sufficient to allow identification with a recognized egg white component, the number of possibilities has been reduced considerably.

Since the inhibitor can be purified at least 62 times on a nitrogen basis, it is evident, on simple assumptions, that the inhibitory activity cannot be associated with any component which contributes more than about 1.6 per cent of the total egg white nitrogen. Ovalbumin, conalbumin, and ovomucoid (ovomucoid-α (21)) are ruled out by this criterion (22); purified ovomucoid (15) was found inactive by direct test. The fast moving component *F*, recently described (23), and components *G2* and *G3* (22) have not yet been isolated for direct study. Crystalline lysozyme (24), identified with *G1* (25), had essentially no inhibitory capacity. Avidin (26, 27) has a sedimentation constant of 4.7 S (27) in contrast with 31 to 37 S for the inhibitor. Semipurified inhibitor at pH 7.2 and 8.6 shows a slow moving electrophoretic component in the globulin-ovomucoid area and a fast moving component with mobility similar to that of the *F* component. Further experiments to clarify the relation of inhibitor to these components are under way.

From another point of view the behavior of the inhibitor during fractionation and the properties of purified inhibitor suggest a possible identity with ovomucin (ovomucoid-β (21)). While such an identification would accord with the properties of virus inhibitors from other sources (2, 4, 17, 28), it must be noted that ovomucin is stated to be completely insoluble after precipitation (21). In contrast, solutions of purified inhibitor were obtained with activities one-third to one-half that of egg white on a volume basis (Tables I and III).

Correlation of Physical Data—Studies of semipurified inhibitor with the ultracentrifuge reveal (Text-fig. 1) a single, slightly diffuse boundary and a sedimentation behavior similar to that of spheres with hydrated density of 1.25 and mean diameter of about 15 mμ. Electron micrographs show spheroidal particles of such size occurring predominantly in groups of indefinite configuration or in threads (Fig. 2). There is little doubt, at present, that the particles observed with the microscope are related to the sedimentable component. Furthermore, studies with the quantity ultracentrifuge (*e.g.*, Table II) indicate that the sedimentable component carries a great part, if not all, of the inhibitory activity; 70 to 80 per cent of the activity is sedimented in 60 to 90 minutes at 67,000 × *g*.

The hypothesis of spheres is inconsistent, however, with evidence from viscosity studies, which indicates that the inhibitory activity is associated with highly asymmetric particles. The specific viscosity of Preparation A178-PIII-EI (Table I, purification factor 41) was linear with concentration from 0 to 150 γ of N per ml. and had the value 0.255 at 100 γ of N per ml. (18). Since, as a first approximation, this specific viscosity can be attributed entirely to the inhibitor, a preparation with purification factor 62 would have a specific viscosity of 0.255 at only 66 γ of N per ml. (*cf.* (18)). Assuming that the inhibitor particles are *rigid* prolate ellipsoids with hydrated density of 1.25, one may calculate a minimal[5] viscosity increment (29) of about 500, an axial ratio of about 90 (30), and a frictional coefficient (f/f_0) of about 3.9 (31); these values are not greatly dependent on the assumed magnitude of hydration. Taken with the sedimentation constant 31 S, the frictional coefficient 3.9 yields a minimal molecular weight[6] of about 7.6 × 10^6 (31), corresponding to rod-like particles with dimensions about 5 mμ wide by 450 mμ long. Particles of such dimensions would be discernible, but they do not appear in the available electron micrographs.

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[5] Minimal because the particles are probably oriented during the measurement of viscosity.

[6] The molecular weight is minimal, since the sedimentation constant has not been corrected for the viscosity due to the solute. Furthermore, the minimal viscosity

The discrepancy between the micrograph data and the predictions from the viscosity-sedimentation data may be accounted for by several suppositions: (*a*) the micrograph images may be artifacts, their shape bearing little relation to the shape of particles suspended in buffer; and (*b*) the inhibitory activity may be associated with flexible rods or branched chains, rather than with rigid rods. The experimental data are inadequate at this time for further speculation on the structure of the particles.

Quantitative Relation between Inhibitor and Virus—With the most active preparations of semipurified inhibitor, 0.004 γ of N is sufficient to inhibit the hemagglutinating activity of 1 hemagglutinating dose of heated influenza virus of swine, corresponding to about 0.04 γ of N of the purified virus preparation. One particle of virus, assumed spherical, with a radius of 58 mμ, density 1.100, partial specific volume 0.850 (32), and nitrogen content of 9.0 per cent of the dry weight (33), contains about 4.9×10^{-17} gm. of N. The amount of inhibitor N needed to neutralize one virus particle is thus about 4.9×10^{-18} gm. One particle of inhibitor, with a molecular weight of 7.6×10^{6} (see above) and nitrogen content of 12.5 per cent, contains about 1.6×10^{-18} gm. of N. Accordingly, one may calculate that approximately three particles of inhibitor are sufficient to neutralize one particle of virus. For this calculation it is assumed that the inhibitor and virus preparations consist entirely of the active ingredients. If the viscosity data are neglected and the inhibitor particles are assumed to be spheres with a hydrated density of 1.25, the molecular weight becomes about 1×10^{6}, and approximately twenty-three particles of inhibitor are needed.

SUMMARY

By means of chemical or ultracentrifugal fractionation, the egg white inhibitor of swine influenza virus hemagglutination has been obtained in solutions up to 60 times as active as crude egg white. The inhibitory activity was associated with a highly viscous, heat stable, non-dialyzable component with the properties of a protein-carbohydrate complex. Analysis showed N 12.5, S 2.4, and carbohydrate, calculated as glucose, 10.3 per cent; no phosphorus was found. An ultraviolet absorption maximum occurred at 2780 A. Electrophoretic analysis showed two components, one corresponding to the *F* component and the other to a member of the globulin-ovomucoid group.

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increment gives a minimal frictional coefficient and, again, a minimal molecular weight.

The active component sedimented to the extent of 70 to 80 per cent in 90 minutes at 67,000 × g, and Lamm scale analysis showed a single, slightly diffuse boundary with a sedimentation constant of 31 to 37 S. Electron micrographs revealed spheroidal particles 10 to 20 mμ in diameter, arranged in clusters of indefinite configuration or in threads. The mean diameter derived from the sedimentation constant on the assumption of spherical particles of hydrated density 1.25 was about 15 mμ. In contrast, classical treatment of the sedimentation and viscosity data together indicated a particle molecular weight of 7.6×10^6 for a rigid prolate ellipsoid with an axial ratio of 1:90. No evidence of such particles was seen in the electron micrographs.

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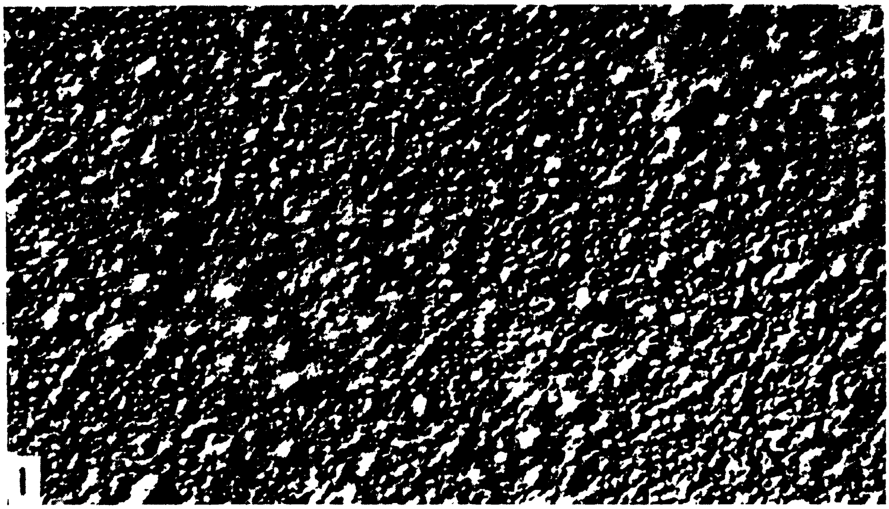
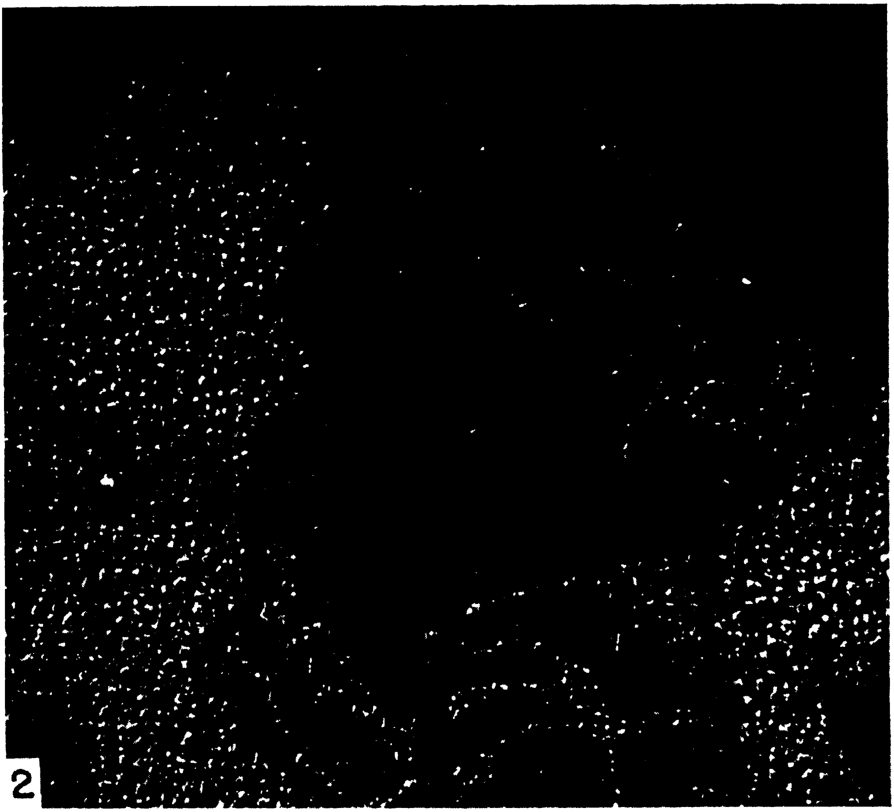
EXPLANATION OF PLATE 4

Fig. 1. Electron micrograph of a preparation (UC-PI, Table II) of egg white inhibitor with a purification factor of 22 (see the text). The preparation, containing 202 γ of N per ml. in 0.06 M phosphate buffer at pH 7.2, was diluted 1:10 with water. Magnification, 60,000 ×.

Fig. 2. Electron micrograph of a preparation (A178-PIII-EI, Table I) of egg white inhibitor with a purification factor of 41. The preparation, containing 148 γ of N per ml. in 0.06 M phosphate buffer at pH 7.2, was diluted 1:100 with water. Magnification, 60,000 ×.



1



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(Lanni, Sharp, Eckert, Dillon, Beard, and Beard: Influenza virus)

STUDIES OF ARTERIOVENOUS DIFFERENCES IN BLOOD SUGAR*

IV. EFFECT OF INTRAVENOUS INSULIN AND SIMULTANEOUS GLUCOSE FEEDING

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This is a report dealing with the effects of insulin upon the rate of peripheral glucose assimilation during alimentary hyperglycemia. Available information on the subject is rather scanty. Cori and Cori (1) showed that in rabbits the arteriovenous differences were nearly 50 per cent greater after glucose feeding and simultaneous insulin injection than after glucose feeding alone. Cori, Pucher, and Bowen (2) observed a similar response in six out of seven diabetic patients. The limited scope and nature of the information concerning this subject, which can be culled from the literature up to 1946, is fairly reflected by a brief résumé by Peters and Van Slyke (3), in which it is stated that "insulin has relatively little effect on alimentary hyperglycemia of normal subjects... It does tend to curtail the hyperglycemia and to exaggerate the terminal hypoglycemic reaction."

We hoped to obtain more detailed facts by means of the procedure and analytical technique which we employed in our previous studies of arteriovenous (A-V) differences (4). Healthy young men served as the subjects in these experiments, each undergoing two tests. First they were fed 50 to 100 gm. of glucose; in the second test from 3 to 5 units of insulin were injected intravenously simultaneously with the feeding of the same amount of glucose as in the first test. Changes in the arterial (capillary) and venous blood sugar levels were then observed at certain intervals in the course of 2 to 4 hours. It may be noted that we adhered to the practice of using rather small insulin doses. This was necessary in order to avoid, as far as possible, hypoglycemic states, the explicit purpose of our studies being the observation of insulin action during hyperglycemia. It will be seen from our results that we were not always successful in preventing hypoglycemia, even when the insulin dose was as small as 3 units.

The results of four of these experiments are presented in Table I. As may be seen, insulin caused marked depression of both the arterial and

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venous blood sugar levels. As a matter of fact, in two subjects (Nos. 1 and 2) the venous blood became hypoglycemic half an hour after the administration of glucose and insulin, while the arterial blood sugar, although substantially lower than in the tests without insulin, had risen

TABLE I

Showing Response of Healthy Men to Insulin Injected Simultaneously with Oral Administration of Glucose

Time after glucose feeding	Subject 1			Subject 2		
	Glucose per 100 cc.		A-V difference	Glucose per 100 cc.		A-V difference
	Arterial blood*	Venous blood		Arterial blood	Venous blood	
	100 gm. glucose by mouth			50 gm. glucose by mouth		
hrs.	*mg.*	*mg.*	*mg. per cent*	*mg.*	*mg.*	*mg. per cent*
0	88.6	86.4	2.2	90.5	86.9	3.6
0.5	162.8	133.7	29.1	164.4	144.2	20.2
1	163.4	132.3	31.1	158.8	149.6	9.2
2	114.5	88.6	25.9	71.8	67.0	4.8
3	63.7	62.1	1.6			
4	79.7	78.3	1.4			
	5 units insulin intravenously, 100 gm. glucose by mouth			3 units insulin intravenously, and 50 gm. glucose by mouth		
0	87.8	81.0	6.8	90.5	86.4	4.1
0.5	110.2	58.1	52.1	96.1	75.6	20.5
1	128.8	111.9	16.9	140.7	132.6	8.1
2	114.8	89.1	25.7	110.7	96.1	14.6
3	84.2	82.6	1.6			
4	92.9	87.8	5.1			
	Subject 4 50 gm. glucose by mouth			Subject 5 100 gm. glucose by mouth		
0	90.2	86.6	3.6	96.1	94.5	1.6
0.5	203.3	178.8	24.5	165.8	142.1	23.7
1	169.6	117.5	52.1	135.1	123.7	11.4
2	62.7	54.3	8.4	141.2	115.3	25.9
3				127.7	110.5	17.2
	4 units insulin intravenously, 50 gm. glucose by mouth			5 units insulin intravenously, 100 gm. glucose by mouth		
0	83.7	79.9	3.8	97.6	95.9	1.7
0.5	117.2	88.6	28.6	78.3	71.6	6.7
1	114.8	87.8	27.0	94.5	74.8	19.7
2	70.2	57.0	13.2	113.4	100.4	13.0
3				120.7	97.4	23.3

* Arterial glycemic levels were determined in capillary (finger) blood.

above the postabsorptive level. Insulin action was strongest in Subject 5, as shown by the hypoglycemic states in both arterial and venous blood throughout an hour after the simultaneous administration of 100 gm. of glucose and 5 units of insulin. From the great depression of alimentary hyperglycemia it is obvious that the rate of over-all assimilation was considerably enhanced by as little as 3 units of insulin. Himsworth, in his brilliant studies dealing with the effect of dietary factors on insulin action (5), obtained similar results with the oral administration of 50 gm. of glucose and simultaneous intravenous injection of from 2.5 to 5 units of insulin.

In regard to the peripheral action of insulin, the central object of these studies, the picture presented by our data was not so clear and simple. In some subjects, at some intervals after injection, the A-V difference showed that insulin enhanced the assimilation rate. In other subjects, however, glucose feeding alone increased the A-V differences to nearly the same extent as did glucose feeding with simultaneous insulin injection, as if insulin had not at all influenced peripheral assimilation. Finally, in some instances smaller A-V differences appeared with than without insulin, conveying the impression that insulin may have inhibited peripheral glucose assimilation. All three of these variations appeared even in a single subject at various time intervals after the injection of insulin.

This is a very confusing picture, indeed, but only until one applies to its interpretation two facts which we presented in previous papers. One of these facts is the close relationship that obtains between the extent of hyperglycemias and the magnitude of A-V differences; we have shown that in any healthy person increasing hyperglycemic levels entail increasing A-V differences (4). The second pertinent fact concerns the influence of hypoglycemia on A-V differences; we have found that hypoglycemia activates an insulin-antagonistic mechanism, with the consequence that peripheral insulin action is counteracted during hypoglycemic states, and this is reflected in a shrinkage of A-V differences (6, 7). Erroneous conclusions can be avoided only if our results are analyzed in the light of these two facts.

In Subject 1 (Table I), for instance, the A-V difference increased to 52.1 mg. per cent in the course of ½ hour after insulin injection, whereas with glucose feeding alone it had gone up to only 29.1 mg. per cent. This difference between the two responses, attesting to a marked action of insulin, is further accentuated by the fact that the greater increase occurred in spite of a lower hyperglycemic level. At the end of the second half hour period the A-V difference has appreciably decreased in the test with insulin. This, however, is the invariable sequel to the emergence of hypoglycemia in the venous blood, a sharp dip to 58.1 mg. per cent, which activated the insulin-antagonistic mechanism, which in turn frustrated peripheral insulin action. This defense reaction against hypoglycemia prevailed only until the hypoglycemic effect of insulin had been successfully combated and the excitation of the insulin antagonists had subsided. This was attained at the end of the 2nd hour, when the blood sugar again rose above the fasting level; at this point the A-V difference showed again a substantial increase.

In a previous paper (7) we stated that it is arterial hypoglycemia that activates the insulin antagonists, while venous hypoglycemia fails to exert such an effect so long as the arterial blood sugar stays above its postabsorptive level. In those experiments, however, which led to this conclusion, the venous hypoglycemias were of rather slight degrees, whereas in the present experiment (Subject 1) the venous blood sugar decreased sharply by 22.9 mg. per cent within ½ hour. Venous hypoglycemias of such substantial degrees apparently do stimulate the insulin-antagonistic mechanism, even while the arterial blood sugar maintains moderate hyperglycemic levels.

That venous hypoglycemia of a minor degree exerts far less of this effect is shown by the response of Subject 2, who received only 3 units of insulin. As may be seen in Table I, this subject also developed venous hypoglycemia ½ hour after insulin injection, but the dip was only 10.8 mg. per cent below the fasting level and apparently caused less shrinkage in the A-V difference than did the more intensive hypoglycemia in Subject 1. It may be noted that the A-V difference at the half hour period in Subject 2 was much the same with and without insulin. If, however, one takes into consideration that in the test with glucose feeding alone a hyperglycemic level of 164.4 mg. per cent was necessary to produce a 20.2 mg. per cent A-V difference, it is evident that in the second test it was the action of the injected 3 units of insulin that produced an A-V difference of the same magnitude, *i.e.* 20.5 mg. per cent, practically without any of the synergistic effect of hyperglycemia. It may be said, then, that the 3 units of insulin alone effected the increase in the rate of peripheral assimilation, since our past experiments indicate that the slight rise of merely 5.6 mg. per cent in the arterial blood sugar that took place in Subject 2 does not measurably affect the A-V difference (4). This experiment shows emphatically the powerful stimulus upon the rate of peripheral glucose assimilation which can be exerted by as little as 3 units of insulin.

Subject 5 deserves especial attention because of his greater sensitivity to insulin (see Table I). In response to 5 units, injected simultaneously with the oral administration of 100 gm. of glucose, he promptly developed hypoglycemia not alone in the venous, but also in the arterial, blood, and the hypoglycemic state persisted through the entire 1st hour. This means that insulin action was so potent that the rate of intestinal absorption of glucose was unable to cope with the rate of assimilation. The resulting hypoglycemia then activated the insulin-antagonistic mechanism. This reaction, as we know, is especially responsive to arterial hypoglycemia (7). As a consequence, the A-V difference increased to only 6.7 mg. per cent at the half hour period, a response resembling that observed after the injection of a similar dose of insulin in the postabsorptive state (6). Small as this increase in the A-V difference is, it still testifies to the fact that insulin action outstripped the antagonistic factors at the half hour period, but the balance was only slightly positive in favor of the insulin. During the second half hour interval, however (*i.e.*, between 30 and 60 minutes after injection), when the arterial blood sugar had nearly reverted to the postabsorptive level, the excitation of the insulin-antagonistic mechanism began to subside and insulin action gained gradual ascendency over it. As a result, the A-V difference increased at the end of this period to the substantial value of 19.7 mg. per cent, despite the continuing absence of arterial hyperglycemia, and despite the persistence of venous hypoglycemia. Insulin action here was quite powerful, more so than that indicated by the A-V difference, since the latter reflects the action of only that fraction of the 5 units which was not counteracted by the antagonistic factors.

The response of Subject 4 (Table I) differed from that of the preceding three in that he developed no hypoglycemia after glucose feeding and the simultaneous injection of insulin. Nor did hyperglycemia reach any substantial degree. In fact, the action of 4 units was potent enough to prevent the venous blood sugar from rising higher than 88.6 mg. per cent at any time during the absorption of 50 gm. of glucose. This is in sharp contrast of 178.8 mg. per cent of venous hyperglycemia that developed after glucose feeding alone. Irrespective of the pronounced insulin action in the body as a whole, however, a distinctly lower peripheral assimilation rate is apparent in Subject 4 with than without insulin, as if insulin had acted as an inhibitor. Unlike the other three cases, no hypoglycemia had occurred in Subject 4; so that the lower A-V differences in the second test are entirely due to the fact that the blood sugar stayed at relatively low levels after insulin injection. As may be noted in Table I, the maximal arterial hyperglycemia after the ingestion of 50 gm. of glucose in this instance was 203.3 mg. per cent, whereas when 4 units of insulin were injected simultaneously with glucose feeding the maximum was only 117.2 mg. per cent. This great discrepancy was the factor which so effectively masked the action of insulin on peripheral assimilation, a factor which requires correction if valid comparison of the two tests is to be made.

While it appears impossible to find a way for a quantitative correction of the effect of the insulin-antagonistic factors, we can offer a formula which permits of correction for differences in the hyperglycemic levels. The correction involves the following steps. In the first place, we add up the A-V differences that were determined at various intervals during alimentary hyperglycemia, and designate this sum as the "peripheral assimilation index." This sum, introduced in a previous study (6), furnishes an integrated picture of the peripheral assimilation for the entire period of the experiment. Likewise we summarize the increment in the arterial glycemic level at the corresponding intervals and denote this sum as the "hyperglycemic sum." For comparison of peripheral assimilation rates obtained in two experiments, the ratios, assimilation index to hyperglycemic sum, are brought to a common denominator. This correlation of the data yields the "relative assimilation index," which permits comparison of two assimilation indices with the elimination of the influence of the difference in the hyperglycemic levels. This calculation is based on the premise that, in any single healthy person, the increase in A-V differences during alimentary hyperglycemia is roughly proportional to the increase in the arterial glycemic level. Hence, if any added factor, as for instance, injected insulin, changes this relationship, the change can be ascribed to the action of this added factor. It is inherent in the nature of physiologic processes that our premise is of limited validity, but calculations on a number of examples convinced us that the relative assimilation index is a useful tool for the evaluation of changes in A-V differences whenever the influence of the hyperglycemic factor is to be discounted in order to permit accounting for the effect of another factor.

An example given in Table II may help to elucidate the meaning and calculation of the relative assimilation index. Two tests are recorded here which were performed on a healthy person. Although identical doses (50 gm.) of glucose were administered in both tests, considerably higher hyperglycemic levels were produced in the second test by injecting part of the glucose (15 gm.) intravenously and feeding by mouth only the remaining 35 gm., whereas in the first test all of the glucose was fed by mouth. It may be seen that, in line with our past observations (4), the higher hyperglycemic levels entailed greater A-V differences. This is expressed in the assimilation index, which in the second test was 75, as against 47 in the first test, a difference of about 70 per cent. Now, if, by applying the formula given in the foot-note below Table II, one reduces the assimilation index of the second test to the same lower hyperglycemic level that prevailed in the first test, one obtains the relative assimilation index for the second test. This figure (Column 7, Table II) means that the assimilation index of the second test would be approximately 41.6 if the hyperglycemic levels were the same as in the first test. Comparison of this relative assimilation index with the actual assimilation index of the first test (which was 47.2), shows a close agreement between the two. (A similar agreement is, of course, in evidence when one calculates the assimilation index of the first test for the hyperglycemic levels of the second test; this figure, 85.2, is fairly close to 75.0, the actual assimilation index of the second test.)

This is the approach we used for the evaluation of insulin action on the rate of peripheral assimilation during alimentary hyperglycemia. For each subject we calculated the relative assimilation index for the first test; *i.e.*, we calculated what the assimilation index would be after glucose feeding alone if the hyperglycemic levels had not risen higher than in the tests in which insulin was injected simultaneously with glucose feeding. The data used in the calculations, and the results, recorded in Table III, illustrate how misleading it would be to evaluate insulin action on the basis of A-V differences without being aware of their relationship to the glycemic levels. From the magnitude of the actual assimilation index (sum of A-V differences), one would conclude that in only two of our five subjects (Nos. 1 and 2) did an increase in the rate of peripheral assimila-

TABLE II

Method of Calculation of Assimilation Index and Relative Assimilation Index

Time after administration of glucose (1)	Arterial blood sugar (2)	Rise above fasting level (3)	A-V difference (4)	Hyper-glycemic sum* (5)	Assimilation index† (6)	Relative assimilation index‡ (7)
50 gm. glucose by mouth						
hrs.	*mg. per cent*	*mg. per cent*	*mg. per cent*			
0	(88.6)	0	(6.0)			
0.5	94.4	5.8	19.4			
1	121.5	32.9	16.2			
2	102.6	14.0	11.6	52.7	47.2	(85.2)
15 gm. glucose intravenously, 35 gm. by mouth						
0	(89.1)	0	(6.5)			
0.5	161.7	72.6	43.2			
1	108.3	19.2	21.9			
2	92.6	3.5	9.9	95.3	75.0	41.6

* Sum of quantities in Column 3.

† Sum of quantities in Column 4.

‡ $75.0 \times \frac{52.7}{95.3} = 41.6$, or $47.2 \times \frac{95.3}{52.7} = 85.2$.

tion occur as a result of insulin injection, and even that increase was insignificant. In one subject (No. 3) there was no change, while in two others (Nos. 4 and 5) insulin seemingly depressed peripheral assimilation. But the picture changes if we consider the relative assimilation index, the value of which is given in the last column of Table III. Comparison of the two tests on this basis shows in every instance a definite enhancement of the peripheral glucose assimilation by injected insulin. When this effect was seemingly absent, it was partly due to the depression of the alimentary hyperglycemia in the tests with insulin injection, and partly to the handicap under which insulin labored when hypoglycemic states led to the mobilization of insulin-antagonistic factors.

TABLE III

Showing That, Relative to Hyperglycemic Levels, Peripheral Glucose Assimilation Is Enhanced by Insulin Injected Simultaneously with Glucose Feeding

Subject No.	Insulin dose	Rise of arterial blood sugar above fasting level, and A-V differences, at intervals of						Hyperglycemic sum	Assimilation index	Relative assimilation index
		0.5 hr.		1 hr.		2 hrs.				
		Blood sugar	A-V	Blood sugar	A-V	Blood sugar	A-V			
	units									
1	None	74.2	29.1	74.8	31.1	25.9	25.9	174.9	86.1	**44.5**
	5	22.4	52.1	41.0	16.9	27.0	25.7	90.4	**94.7**	
2	None	73.9	20.2	68.3	9.2	−18.7	4.8	142.2	34.2	**17.6**
	3	5.6	20.5	50.2	8.1	20.2	14.6	76.0	**43.2**	
3	None	92.6	31.4	105.3	49.2	24.5	29.1	222.4	99.7	**84.4**
	5	97.0	45.1	49.5	26.7	41.7	27.3	188.2	**99.1**	
4	None	113.1	24.5	79.4	52.1	0.4	8.4	192.9	85.0	**28.5**
	4	33.5	28.6	31.1	27.0	−13.5	13.2	64.4	**68.8**	
5	None	69.7	23.7	39.0	11.4	45.1	25.9	153.8	61.0	**6.3**
	5	−19.3	6.7	−3.1	19.7	15.8	13.0	15.8	**39.4**	

SUMMARY

Alimentary hyperglycemia of healthy persons is effectively suppressed by insulin when injected intravenously and simultaneously with oral glucose feeding. Doses as small as from 3 to 5 units may cause hypoglycemic states in subjects who, without injected insulin, develop substantial degrees of hyperglycemia after ingestion of the same amount of glucose. This fact indicates that the physiological insulin requirement of healthy persons is very small.

Insulin effect on the rate of peripheral assimilation during alimentary hyperglycemia cannot be judged on the basis of A-V differences (and the assimilation index), without taking into account two factors: (1) the insulin-antagonistic sequel of hypoglycemia, and (2) the functional relationship between hyperglycemic levels and A-V differences. The first of these two factors is not amenable to measurement. An approximately quantitative correction for the influence of the hyperglycemic factor, however, is feasible. If such a corrected quantity, designated as the "relative assimilation index," is used for evaluation of A-V differences, it was found that insulin invariably enhances the rate of peripheral glucose assimilation when injected intravenously simultaneously with glucose feeding.

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THE REACTION OF CATALASE AND CYANIDE*

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Previous work has suggested that the inhibition of catalase activity by cyanide is of the non-competitive type. This is remarkable, because both cyanide and peroxide are considered to combine with the iron atom of a hemoprotein. In this paper, the mechanism of the cyanide inhibition of catalase is studied in detail. First, kinetic and equilibrium measurements show that the reaction of catalase and cyanide is in accordance with the law of mass action and that the three or four catalase hematins act independently. Second, an improved method for the determination of catalase activity (1) has been used to measure the inhibition of catalase activity by cyanide. The dissociation constants of catalase cyanide calculated from the data obtained in these two cases are in agreement and verify non-competitive inhibition. This result is explained by the assumption that only a portion of the three or four catalase hematins is bound to peroxide during the destruction of hydrogen peroxide. This assumption is strongly supported by the recent finding of the intermediate compound of catalase and hydrogen peroxide, which probably has only one hematin bound to hydrogen peroxide (2).

The non-competitive inhibition of catalase activity by cyanide is in striking contrast to the analogous studies of the cyanide inhibition of peroxidase activity in which classical competitive inhibition was demonstrated in detail (3). Peroxidase, however, has only one hematin group and does not catalyze the decomposition of hydrogen peroxide into water and oxygen.

The reaction kinetics of the formation and dissociation of catalase cyanide have been studied spectrophotometrically by the rapid flow technique. The dissociation constant of catalase cyanide calculated from these kinetic data is in agreement with that obtained from titration and activity data. The velocity of combination of catalase and cyanide is not affected by the presence of hydrogen peroxide (4).

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* This is Paper 1 of a series on catalases and peroxidases.

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The small effect of varying pH upon the dissociation constant of catalase cyanide indicates that the reaction studied under these conditions is

$$\mathrm{Cat(OH)_4 + 4HCN \underset{k_6}{\overset{k_5}{\rightleftarrows}} Cat(CN)_4 + 4H_2O} \tag{1}$$

in which the hydroxyl group found by Agner and Theorell (5) is replaced by the cyanide ion. Since it is found here that the four hematin groups of blood catalase act independently, the reaction is written,

$$\mathrm{FeOH + HCN \underset{k_6}{\overset{k_5}{\rightleftarrows}} FeCN + H_2O} \tag{2}$$

For these purposes the concentration of water is omitted and the apparent dissociation constant is conveniently evaluated on a hematin iron basis as follows:

$$K_I = \frac{[\mathrm{HCN}][\mathrm{FeOH}]}{[\mathrm{FeCN}]} \tag{3}$$

The dimensions of K_I are moles per liter.

Preparations—The catalase preparations were purified by Dr. R. K. Bonnichsen, to whom many thanks are due. Their optical densities were measured spectrophotometrically in the Beckman spectrophotometer, and their concentrations are calculated from the extinction coefficients, $\epsilon_{405} = 340$ cm.$^{-1}$ × mM^{-1} for the horse liver catalases, and $\epsilon_{405} = 380$ cm.$^{-1}$ × mM^{-1} for the horse blood catalases (1). The cyanide solutions were freshly made up before the experiments, but were found to be reasonably stable for a few days as determined from titrations by Liebig's method.

Soret Band of Catalase Cyanide—The Soret band of catalase cyanide can be measured in the Beckman spectrophotometer (6, 7) and has a peak at 425 mμ where $\epsilon = 319$ cm.$^{-1}$ × mM^{-1} for erythrocyte catalase and 274 cm.$^{-1}$ × mM^{-1} for a three-hematin liver catalase. Catalase and catalase cyanide are isosbestic (have the same extinction coefficient) at 417 mμ. Catalase and catalase hydrogen peroxide are isosbestic at 435 mμ (2), and, because some measurements are made in the presence of hydrogen peroxide, this wave-length is used for kinetic studies.

Velocity Constant for Combination of Catalase and Cyanide—The reactions represented by Equation 1 are fairly fast but can be measured by the rapid flow apparatus (8). Since later data show that k_6 of Equation 1 is about 3 sec.$^{-1}$, k_5 can only be measured without correction for k_6 by using a large excess of cyanide, and this precaution has been observed, as shown by the data of Table I. Thus k_5 is found to be 9×10^5 M^{-1} × sec.$^{-1}$. In the presence of hydrogen peroxide, k_5 is found to be 8×10^5 M^{-1} × sec.$^{-1}$.

Velocity Constant for Dissociation of Catalase Cyanide—The reaction of silver ion with cyanide

$$Ag^+ + 2CN^- \rightleftarrows Ag(CN)_2 \qquad (4)$$

has been utilized to reduce the cyanide concentration rapidly and hence to cause the dissociation of catalase cyanide. A buffered catalase solution is saturated with cyanide and then is mixed with less than an equivalent of unbuffered silver nitrate solution in the rapid flow apparatus. The decrease in the concentration of catalase cyanide is followed spectrophotometrically at 425 mμ. Table II shows that the velocity constant, 3.2 sec.$^{-1}$, is very nearly independent of the silver ion concentration. Thus the limiting step in the reaction is the dissociation of catalase cyanide and the average values of the velocity constant, k_6, are 3.2 sec.$^{-1}$ at pH 7.0 and 2.9 sec.$^{-1}$ at pH 4.6. This very large increase of hydrogen ion concentration has a nearly negligible effect upon k_6, and the formation of water according to Equation 2 is thereby substantiated.

TABLE I

Velocity Constant for Formation of Catalase Cyanide in Presence or Absence of Hydrogen Peroxide

1.1 μM of Fe horse liver catalase, 0.01 M phosphate buffer, pH 6.5; λ = 435 mμ (Experiments 82a and 82b).

Initial cyanide concentration, *μM*	50	200	20	50	200
" hydrogen peroxide concentration, *μM*	0	0	10	10	10
Reaction velocity constant, k_5, $M^{-1} \times sec.^{-1} \times 10^{-15}$	9.0	9.0	9.0	8.0	7.5

TABLE II

Velocity Constant for Dissociation of Catalase Cyanide

0.54 μM of catalase and 10 μM of cyanide; λ = 425 mμ (Experiment 317).

	0.01 M phosphate, pH 7.0			0.01 M acetate, pH 4.6		
Silver ion concentration, *μM*	5	10	20	4	10	20
k_6, *sec.*$^{-1}$	3.6	3.3	2.8	2.8	2.6	3.3

The dissociation constant of catalase cyanide is $k_6/k_5 = 3.2/(9 \times 10^5) = 3.6 \times 10^{-6}$ M, in fair agreement with the values obtained from spectrophotometric and activity data in the following experiments.

Dissociation Constant of Catalase Cyanide—The changes of optical density at 425 mμ upon the addition of measured amounts of cyanide to catalase have been measured in the Beckman spectrophotometer. The relation between the experimental values of free catalase (FeOH), catalase cyanide (FeCN), and hydrocyanic acid (HCN) is shown by the 45° straight line of Fig. 1 to be in accordance with the logarithmic form of Equation 3,

$$\log \frac{[\text{FeOH}]}{[\text{FeCN}]} + \log [\text{HCN}] = \log K_I \tag{5}$$

The dissociation constant (K_I) is 4×10^{-6} M with one cyanide bound to each hematin group. These experiments have been carried out over as wide a range of values of FeOH/FeCN as the accuracy of the spectrophotometer permits in order to obtain any evidence of interaction between the four catalase hematins. Over the range from about 7 to 98 per cent saturation of catalase with cyanide there is no change of the dissociation constant of catalase cyanide in excess of the experimental error, and thus there is no detectable heme-heme interaction.

TABLE III

Effect of Change from pH 7.0 to 4.6 upon Dissociation Constant of Catalase Cyanide

$\lambda = 425$ mμ (Experiment 285).

	Corrected optical density of catalase cyanide				
	Cyanide added				
	0 μM	3.3 μM	6.7 μM	10 μM	20 μM
pH 7.0, 0.01 M phosphate	0.087	0.111	0.130	0.138	0.148
" 4.6, 0.001 " "	0.082	0.112	0.131	0.137	0.144

In accordance with tests which showed that the dissociation constant of peroxidase cyanide is independent of pH from 4.2 to 6.2 (3), Table III shows that the dissociation constant of catalase cyanide is also independent of pH in the region 4.6 to 7.0. In this experiment, cyanide was added to two catalase solutions of nearly identical concentration at pH 4.6 and 7.0. At pH 4.6, sufficiently dilute phosphoric acid was used to avoid forming the catalase-phosphate complex (5). In a similar experiment, K_I is found to be about 3 times greater at pH 9.3 than at pH 7.0.

The competition between formate and cyanide for catalase hematin is clearly shown by the data of Table IV. At pH 4.0, formate has a high affinity for catalase ($K = 10^{-5}$ M (5)) and decreases the amount of catalase cyanide. This competition shows that cyanide and formate attach to the same place on catalase hematin. At pH 7.0, the affinity of formate for catalase is much less ($K = 7 \times 10^{-3}$ M (5)) and the concentrations of formate employed cause no effect upon catalase cyanide.

Effect of Cyanide upon Destruction of Hydrogen Peroxide by Catalase—

A modified technique for measuring catalase activity with a minimum of inactivation has been used to redetermine the dissociation constant of catalase cyanide from activity data.

The extinction coefficient of hydrogen peroxide at 215 mμ is sufficiently large to permit the measurement of the disappearance of a few mM with good accuracy in the Beckman spectrophotometer. Dilute peroxide (~ 1 mM) gives a few very small bubbles of oxygen at the end of the reaction, which cause no significant error. The optical density of the dilute catalase solution employed in the activity test ($\sim 1 \times 10^{-9}$ M) is so small that it is negligible. The spectrophotometer is allowed to run for about 15 minutes or more so that the light intensity and the "dark current" (amplifier plate current) are not drifting rapidly and do not require adjustment during the kinetic test.

TABLE IV

Competition between Cyanide and Formate for Catalase Hematin

0.86 μM of horse blood catalase and 130 μM of cyanide; λ = 425 mμ (Experiment 321).

	Corrected optical density				
	Formate added				
	0 μM	65 μM	195 μM	510 μM	1720 μM
pH 7.0, 0.01 M phosphate	0.257	0.258	0.259	0.260	0.258
" 4.0, 0.005 " acetate	0.267	0.258	0.228	0.202	0.190

In inhibition studies, the catalase and cyanide are added to the cuvette, and the spectrophotometer is adjusted. The peroxide is delivered onto a stirring rod and is rapidly stirred into the cuvette, and the stop-watch is started. Readings of the optical density of the hydrogen peroxide solution are taken every 10 or 15 seconds for the 1st minute. It is desirable to "track" the density change continuously and to read off the density values at the appropriate intervals. The final value of density is measured and subtracted from each reading of optical density. The reaction velocity constant is calculated from the formula

$$k_1 = \frac{2.3}{e(t_2 - t_1)} \log \frac{x_1}{x_2} \qquad (6)$$

where k_1 is the velocity constant for the destruction of hydrogen peroxide by catalase, e is the catalase concentration in moles per liter, x_1 is the optical density at t_1, and x_2 is the optical density at t_2. This form of the equation is used because the value of optical density at $t = 0$ cannot be obtained when the hydrogen peroxide is added last.

This method has much to recommend it, since it is much more convenient and just about as accurate as the titrimetric method described previously (1). The value of k_1 for horse blood catalase at 25° is the same according to both methods (3.5×10^7 M^{-1} $\times$ sec.$^{-1}$).

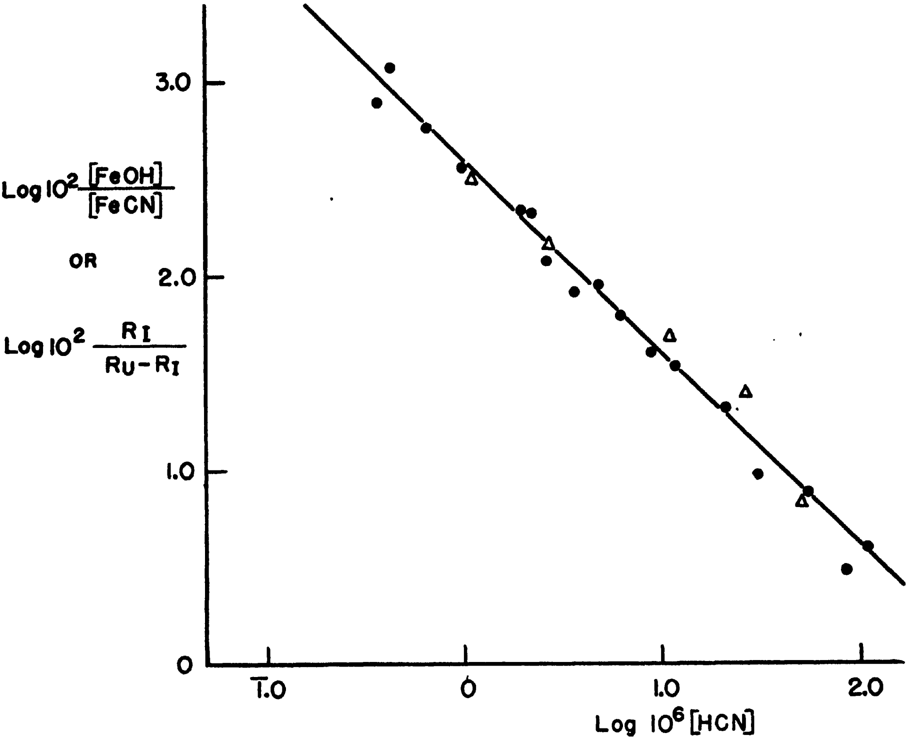

FIG. 1. The dissociation constant of catalase cyanide, $K_I = [\text{FeOH}] [\text{HCN}]/[\text{FeCN}]$, determined from spectrophotometric data (●) and inhibition data (△). The spectrophotometric data were obtained in the Beckman spectrophotometer with a wave-length of 425 mμ and 0.9 μM of horse blood catalase. The inhibition data were determined from the kinetics of hydrogen peroxide disappearance measured at 215 mμ in a solution containing 1 mM of hydrogen peroxide and 1×10^{-9} M horse blood catalase. R_U, the uninhibited rate, $= 3.5 \times 10^7$ M^{-1} sec.$^{-1}$. R_I is the inhibited rate. All experiments were made in 0.01 M phosphate buffer, pH 7.0 at 25° (Experiment 315).

The dissociation constant of catalase cyanide is calculated from the inhibition data by the formula

$$K_I = \frac{R_I[\text{HCN}]}{R_U - R_I} \qquad (7)$$

which has been used previously in studies of peroxidase inhibition (3). R_U is the uninhibited rate (3.5×10^7 M^{-1} $\times$ sec.$^{-1}$) and R_I is the inhibited rate. The data are plotted by using the logarithmic form of Equation 7, and are represented in Fig. 1. These points agree very well with the spectrophotometric data: the dissociation constant of catalase cyanide obtained from spectrophotometric data (4×10^{-6} M) is very nearly identical with the dissociation constant obtained from the inhibition data (4.7×10^{-6} M).

DISCUSSION

The velocity constant for the combination of catalase and cyanide (9×10^{5} $\text{M}^{-1} \times \text{sec.}^{-1}$) and the dissociation constant of catalase cyanide (4×10^{-6} M) are similar to the corresponding values for peroxidase cyanide (1×10^{5} $\text{M}^{-1} \times \text{sec.}^{-1}$, 4×10^{-6} M (3)), although cyanide combines with and dissociates from peroxidase more slowly than from catalase.

The measured values of the dissociation constant of catalase cyanide are not altered over the range from 7 to 98 per cent saturation of catalase with cyanide, and therefore there is no detectable heme-heme interaction in the binding of cyanide to the three hematins of liver catalase. This is in accord with the results of later tests with methyl hydrogen peroxide (9).

In their "magnetic titrations" of catalase with cyanide, Theorell and Agner (10) noted that the curves of magnetic susceptibility *versus* cyanide concentration (*cf.* (10), Figs. 1 and 2) did not follow a straight line but were bent before saturation of catalase with cyanide ion occurs and attributed this effect to heme-heme interaction: cyanide ion bound to 1 or 2 of the 3 hematin iron atoms alters the magnetic susceptibility of the free hematin iron atoms. But the curvature they found is caused by the dissociation of catalase cyanide. Their data follow the theoretical titration curve expected for 600 μM of hematin iron catalase and $K_I = 3 \times 10^{-6}$ M. Therefore, their magnetic data support the conclusion that there is no detectable heme-heme interaction in catalase cyanide.

The nature of the reaction of catalase and cyanide as given in Equation 1 is verified by these experiments. In the equilibria for catalase hydroxide and hydrogen cyanide,

$$\text{FeOH} \longleftrightarrow \text{Fe}^+ + \text{OH}^-, K = 10^{-10.2} \qquad (8)^1$$

$$\text{HCN} \longleftrightarrow \text{H}^+ + \text{CN}^-, K = 10^{-8.7} \qquad (9)^2$$

the product [Fe] [CN⁻] is nearly constant over the range pH 8.7 to 3.8 (14 − 10.2); over this pH region [Fe⁺] is increasing as rapidly as [CN⁻] is decreasing. Both [Fe⁺], and [CN⁻] are so small that [FeOH] and [HCN] are nearly constant. This has been verified by the experimental data which show no increase of K_I at pH 4.6 and some increase at pH 9.3 compared with the values found at pH 7.0.

[1] See Agner and Theorell (5).

[2] See Coryell, Stitt, and Pauling (11).

This reaction of catalase hydroxide and hydrocyanic acid differs from that found by Coryell, Stitt, and Pauling (11) in their studies of ferrihemoglobin cyanide. First, the dissociation constants of the heme-linked hydroxyl groups differ ($10^{-8.12}$ for ferrihemoglobin and $10^{-10.2}$ for catalase). Second, the effect of the hydroxyl group upon the spectra of these hemoproteins is quite different; no shift has yet been detected in the catalase spectrum due solely to the formation of catalase hydroxide, while the shift from acid to alkaline ferrihemoglobin gives an obvious color change. Nevertheless, the data of Agner and Theorell clearly show that the hydroxyl group in catalase can be displaced by anions which do give a spectrophotometrically detectable effect and which inhibit catalase activity. Therefore catalase has a heme-linked hydroxyl group (5).

Further proof that the hydroxyl group is attached to catalase hematin is given by the competition between formate and cyanide at pH 4.0. Thus cyanide and formate appear to attach to the same place on catalase hematin, which, in the case of cyanide, is recognized to be at the iron atom of catalase (10). Therefore the hydroxyl and formate ions also attach to the iron atom.

The excellent agreement between the determinations of the dissociation constant of catalase cyanide from spectrophotometric and from activity data, typical of non-competitive inhibition, confirms the relatively crude tests of Zeile and Hellström (12). This result is a distinct contrast to the results obtained in the cyanide inhibition of peroxidase activity (3).

The non-competitive inhibition of the activity of catalase by cyanide has been recognized for some time, and the only suitable explanation that could be offered previously in view of the then accepted "Michaelis constant" (~0.025 M) for catalase activity was that peroxide and cyanide did not combine at the same place on catalase hematin (13). This "Michaelis constant" for catalase activity has recently been shown to be an artifact due to catalase inactivation during the kinetic test (1).

An alternative explanation is that catalase hematins are not all bound to hydrogen peroxide during the destruction of hydrogen peroxide. The free catalase hematins can then combine with cyanide in a "non-competitive" manner.[3] This explanation is in accord with these experiments and with the properties of the intermediate compound of catalase and hydrogen peroxide which has recently been discovered (2). Both titration and spectrophotometric data (2) suggest that not all catalase hematins are bound to peroxide in this complex. Inhibition of catalase activity may be caused by the combination of cyanide with these free catalase hematins according to Equation 10.

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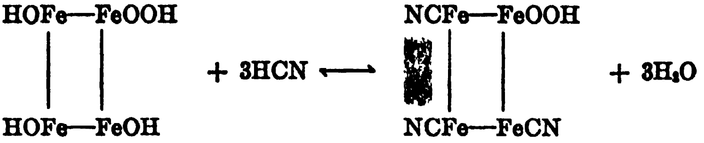
[3] The usual concepts of competitive and non-competitive inhibition have been evolved from considerations of the simple Michaelis theory. However, it has been suggested that the mechanism by which catalase decomposes hydrogen peroxide into oxygen and water involves consecutive reactions of hydrogen peroxide with catalase and with the catalase-hydrogen peroxide complex (14). In such a reaction, the steady state concentration of the catalase-hydrogen peroxide complex may not reach a value corresponding to all hematin groups bound to peroxide and may be quite independent of the hydrogen peroxide concentration. For this reason, the inhibition catalase activity by cyanide will be independent of peroxide concentra-

TABLE V

Summary of Determinations of Dissociation Constant of Catalase Cyanide on Basis of Activity Data

	Titrimetric	Titrimetric	Titrimetric	Titrimetric	Manometric	Manometric	Rapid spectrophotometric
Temperature, °*C.*	0	0	0	0	18	0	25
Initial hydrogen peroxide concentration, *mM*	4	1.8-9.0	5	10	4.5	5.0	1.0
Dissociation constant, *M* × 10^6	1.0	1.0	0.8	6.3	4.6	3.4	4.7
Reference	Wieland (15)	Von Euler and Josephson (13)	Ziele and Hellström (12)	Stern (16)	Keilin and Hartree (17)	Lemberg and Foulkes (18)	This paper



$$\begin{array}{c} \text{HOFe—FeOOH} \\ |\quad\;\; | \\ \text{HOFe—FeOH} \end{array} + 3\text{HCN} \rightleftarrows \begin{array}{c} \text{NCFe—FeOOH} \\ |\quad\;\; | \\ \text{NCFe—FeCN} \end{array} + 3H_2O \qquad (10)$$

These catalase hematins which become blocked by cyanide in this manner must have been active in the destruction of hydrogen peroxide; otherwise, no inhibition would be caused by the reaction of Equation 10.

The results of several determinations of the dissociation constant of

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tion, "non-competitive;" in spite of the fact that both cyanide and peroxide can combine with catalase hematin, they need not compete for the same catalase hematin group in order to inhibit catalase activity. On the other hand, direct competition between peroxide and cyanide for the same hematin group will occur at cyanide concentrations greater than those required to inhibit the activity, and this reaction is studied spectrophotometrically in detail (see Chance (4)).

catalase cyanide based on activity studies are summarized in Table V. It is clear that the more recent determinations with pure catalase give consistently higher values than those obtained by the earlier workers who used cruder preparations (with the exception of Stern). This effect is not due to temperature alone, because Lemberg and Foulkes' data taken at 0° agree with Keilin and Hartree's and these data. Apparently the purer catalases are less sensitive to cyanide than are the cruder solutions.

SUMMARY

1. The velocity constant for the combination of catalase and cyanide is $9 \times 10^5 \times \text{sec.}^{-1}$. The velocity constant for the dissociation of catalase cyanide is 3.2 sec.^{-1} at pH 7.0 and 2.9 sec.^{-1} at pH 4.6. The dissociation constant of catalase cyanide (K_I) calculated from kinetic data is 3.6×10^{-6} M.

2. On the basis of spectrophotometric data, $K_I = 4 \times 10^{-6}$ M.

3. By an improved method for determining catalase activity, it is found that $K_I = 4.7 \times 10^{-6}$ M, on the basis of the cyanide inhibition of catalase activity.

4. The non-competitive inhibition of catalase by cyanide is due to the fact that not all catalase hematins are bound to peroxide in the catalase-hydrogen peroxide complex.

5. Over the range $3.8 < \text{pH} < 8.7$, the reaction of catalase with cyanide is represented by the equation

$$\mathrm{FeOH + HCN} \underset{k_6}{\overset{k_5}{\longleftrightarrow}} \mathrm{FeCN + H_2O}$$

The four hematins of an erythrocyte catalase act independently. The apparent dissociation constant is calculated according to the relation

$$K_I = \frac{[\mathrm{FeOH}][\mathrm{HCN}]}{[\mathrm{FeCN}]}$$

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THE COMPOSITION OF CATALASE-PEROXIDE COMPLEXES*

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Recently the long sought intermediate compound of catalase and hydrogen peroxide has been identified, and its spectrum and reaction kinetics have been measured (1, 2). The properties of this complex are not, however, those of a classical enzyme-substrate compound of Michaelis and Menten. One very unusual property of this complex is that not all of the four catalase hematins of erythrocytes are bound to peroxide, even when saturation with peroxide is demonstrated spectrophotometrically. It is the purpose of this investigation to study this property of the catalase-hydrogen peroxide complex in some detail and to obtain a quantitative measure of the number of peroxide molecules bound to the catalase molecule.

Several independent experimental methods indicate this property of the complex in a qualitative manner. First, titration of catalase with hydrogen peroxide gives molar amounts of this complex in excess of the molar amount of peroxide added when the calculation is based upon the combination of peroxide with all the catalase hematins. Second, the non-competitive inhibition of catalase activity by cyanide (3) indicates that not all catalase hematins are combined with peroxide under the conditions of the test for catalase activity. Third, the spectrum of catalase hydrogen peroxide is very unusual. The difference of extinction coefficient between catalase and this catalase-hydrogen peroxide complex is rather small, 40 $\text{cm.}^{-1} \times \text{mM}^{-1}$ at 405 mμ, which is about equal to the difference between the extinction coefficients of a four-hematin horse erythrocyte catalase (380 $\text{cm.}^{-1} \times \text{mM}^{-1}$) and a three-hematin horse liver catalase (340 $\text{cm.}^{-1} \times \text{mM}^{-1}$) (1).

On the other hand, the change of extinction coefficient between catalase and the unstable primary catalase-alkyl hydrogen peroxide complexes is large (180 $\text{cm.}^{-1} \times \text{mM}^{-1}$ at 405 mμ for horse erythrocyte catalase) (2). A possible explanation of the greater change of extinction coefficient in the latter reaction may be that more alkyl hydrogen peroxide groups than hydrogen peroxide groups attach to catalase hematins. In fact, on the assumption that the change of extinction coefficient per peroxide group bound to hematin iron is the same for alkyl hydrogen peroxides and for hydrogen peroxide, the spectral data suggest that 4 times as many alkyl hydrogen groups as hydrogen peroxide groups attach to catalase; *i.e.*, four alkyl hydrogen groups per erythrocyte catalase molecule, but only one hydrogen peroxide group per catalase molecule.

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* This is Paper 2 of a series on catalases and peroxidases.

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It is of importance to obtain further proof of such a difference in the nature of these compounds, because it may lay the foundation for an explanation of the tremendous difference in the reactivities of catalase towards hydrogen peroxide compared with alkyl hydrogen peroxides. Catalase decomposes hydrogen peroxide into oxygen and water with a tremendous velocity (in 0.1 M hydrogen peroxide the turnover number is $3.5 \times 10^7 \times 0.1 = 3.5 \times 10^6$ times per second (Bonnichsen, Chance, and Theorell (4))), while catalase scarcely decomposes the alkyl hydrogen peroxides at an appreciable velocity in the absence of alcohols (a turnover number of about 0.02 times per second).

Previous methods of determining the number of peroxide groups attached to hematin (for example, those used by Keilin and Hartree (5) in the studies of the methemoglobin-hydrogen peroxide and ethyl hydrogen peroxide complexes) are completely unsuitable here, since not only is the catalase-hydrogen peroxide complex fairly unstable, but a considerable amount of hydrogen peroxide is decomposed during its formation (1).

Even the rapid spectrophotometric titration used in the study of the primary peroxidase-hydrogen peroxide complex (6) is unsatisfactory here, since the mechanism of hydrogen peroxide decomposition must be completely formulated in order to use such titration data in a calculation of the number of hydrogen peroxide groups bound to catalase hematin. It is desirable to use a purely chemical test for unbound hematin groups in catalase-peroxide complexes which requires no postulates of enzymatic mechanism.

If a catalase-peroxide complex is mixed, for example, with cyanide, which reacts very rapidly with free catalase hematins (3), the initial amount of the catalase cyanide compound formed relative to that formed from free catalase is a direct measure of the number of the free catalase hematin groups in the catalase-peroxide complex. The amount of catalase-cyanide complex is readily estimated spectrophotometrically. Some of the characteristics of the catalase-cyanide reaction are now reviewed to determine whether cyanide is a suitable reactant.

Reactions of Catalase with Cyanide or Peroxide—The chemical method for determining the number of free hematin groups in a catalase-peroxide complex requires that cyanide and peroxide compete for the same place on the iron atoms of catalase. There is, however, no direct evidence of competition between hydrogen peroxide and cyanide in the inhibition of the destruction of hydrogen peroxide (3), although competitive inhibition can be completely demonstrated for peroxidase (7). In this paper, direct spectroscopic evidence is presented that cyanide can displace hydrogen peroxide from catalase hematin (see Fig. 2), and therefore cyanide is a suitable substance in this respect. Thus the lack of competitive inhibition of catalase is caused by the peculiar mechanism of the catalase-hydrogen peroxide reaction and not by the fact that cyanide and peroxide combine at different parts of the hematin group.

Direct evidence that cyanide forms a ferric iron covalent compound with catalase is furnished by the magnetic measurements of Theorell and Agner (8). From direct studies of the reaction of catalase and cyanide (3) and from analogy with studies of methemoglobin cyanide (9), it is clear that cyanide ion combines with catalase iron. Magnetic studies (8) further indicate that cyanide ion combines only with intact catalase hematins and not with those partially or completely converted into bile pigment; a three-hematin horse liver catalase molecule binds only 3 cyanide ions.

Methods

Since the catalase-hydrogen peroxide complex begins to decompose several tenths of a second after its formation (see Chance (1), especially Fig. 3), the rapid flow technique (10) is essential.

The ideal form of the rapid flow method for this purpose is the two-mixer technique of Hartridge and Roughton (11), in which the unstable catalase peroxide is formed in one mixing chamber and, at any short time later, is mixed with cyanide ion in a second mixing chamber. However, completely satisfactory results may be obtained with a single mixing chamber in the following general cases. (1) Catalase is mixed with peroxide and cyanide simultaneously, but the peroxide reaction is complete before the cyanide reaction has progressed appreciably. (2) The catalase-peroxide complex is stable enough to permit premixing in a test-tube, followed by reaction with cyanide in the rapid mixing chamber.

The first method is applicable to the catalase-hydrogen peroxide complex and the second to the catalase-alkyl hydrogen peroxide complexes. The kinetic and equilibrium data for the catalase-cyanide and catalase-peroxide complexes specify the relative cyanide and peroxide concentrations for a successful experiment. The following equations define the reaction velocity constants involved in the kinetics and equilibrium. They are written for the specific case of a three-hematin horse liver catalase and a catalase-hydrogen peroxide complex in which only one of the three hematins is bound. Equation 1 is similar to that given previously, except that the value of $n = 1$ is used here (1), and the heme-linked hydroxyl group of catalase is indicated (12). The definition of the velocity constants and the equation for the reaction of catalase and cyanide were given previously (3). It is assumed that the catalase hematins react independently.

$$\mathrm{Cat(OH)_3 + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftarrows}} Cat(OH)_2(OOH) + H_2O} \tag{1}$$

$$\mathrm{Cat(OH)_2(OOH) + 2HCN \underset{k_6}{\overset{k_5}{\rightleftarrows}} Cat(CN)_2(OOH) + 2H_2O} \tag{2}$$

The initial hydrogen peroxide concentration is x_0, and the initial cyanide concentration is i_0. The values of k_1 and k_5 are 3×10^7 M^{-1} sec.$^{-1}$ (1) and 9×10^5 M^{-1} sec.$^{-1}$ (3) respectively. The value of k_5 is very slightly affected by whether or not peroxide is already bound to a catalase hematin. The apparent dissociation constant for Equation 2 is 4×10^{-6} M (3). The apparent dissociation constant for Equation 1 is about 1×10^{-6} M (1).

Method 1—If the composition of the complex of Equation 2 is to be determined to an accuracy of 3 per cent, the velocity of formation of the hydrogen peroxide complex (k_1x_0) must be at least 30 times greater than the velocity of formation of the cyanide complex (k_5i_0); otherwise, cyanide might compete with hydrogen peroxide.

$$\frac{k_1 x_0}{k_5 i_0} > 30; \frac{3 \times 10^7 x_0}{1 \times 10^6 i_0} > 30; \therefore x_0 > i_0 \tag{3}$$

Thus the initial hydrogen peroxide concentration need be only slightly greater than the cyanide concentration.

Since catalase must be saturated with hydrogen peroxide, the initial hydrogen peroxide concentration must be larger than 10^{-6} M.

The initial cyanide concentration should be large enough to give a readily measurable amount of the catalase cyanide compound. In this particular method, it has been convenient to use an initial cyanide concentration which gives about 0.5 to 0.75 saturation of catalase with cyanide. When the catalase concentration is comparable to the cyanide concentration, there are appreciable differences in the concentration of uncombined cyanide, depending upon whether the reaction is with free catalase or with the catalase-hydrogen peroxide complex, and appropriate corrections to the concentration of catalase cyanide are calculated in the "Appendix."

Method 2—The catalase-alkyl hydrogen peroxide complexes are stable for about a minute, and therefore the complexes, preformed in a test-tube, are mixed with cyanide in the rapid flow apparatus.

Spectroscopic Considerations—The primary catalase-peroxide complexes and catalase have an isosbestic point (the same extinction) at 435 mμ, and therefore the concentration of the catalase-cyanide complex is measured at this wave-length.

Choice of Catalases—If hydrogen peroxide is bound to only one catalase hematin, a 25 per cent decrease in the amount of catalase cyanide compound formed on reaction of cyanide with the catalase-hydrogen peroxide complex would be expected with a four-hematin erythrocyte catalase and a 100 per cent decrease would be expected with a one-hematin catalase, in accordance with Equation 2 above. Thus the method is most sensitive with low hematin catalases. Although low hematin catalases may be prepared by the methods of Lemberg and Legge (13), the methods for determination of the bile pigment content require large corrections. The hematin content of horse liver catalase prepared according to the methods of Agner (14) and Bonnichsen (15) has been determined by several methods, including splitting experiments, magnetic titrations (8), and spectrophotometric tests (3, 15), and appears to be rather close to three intact hematins. However, experiments with the four-hematin catalase of horse erythrocytes and with an especially prepared low hematin catalase are described. The catalases used in these experiments were purified by Dr. R. K. Bonnichsen, to whom many thanks are due.

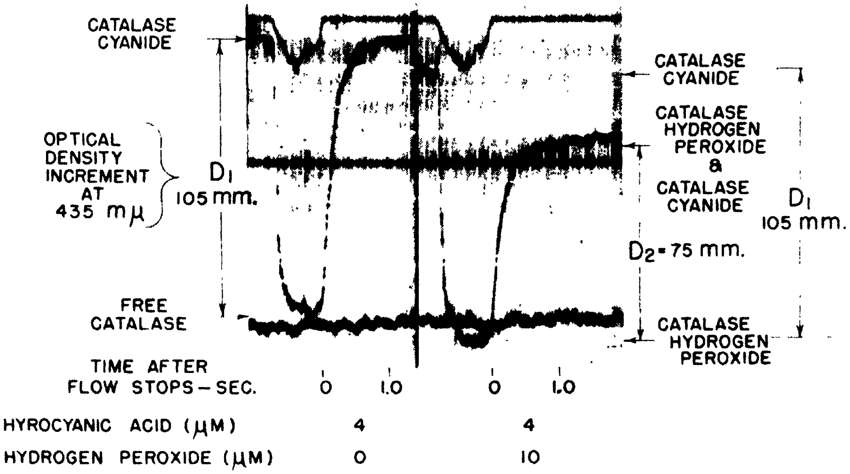

FIG. 1. The kinetics of the formation of catalase cyanide in the absence (left-hand) and in the presence (right-hand) of hydrogen peroxide. 3.4 μM of hematin iron horse liver catalase, pH 6.5, 0.01 M phosphate buffer (Experiment 82b).

Composition of Catalase Hydrogen Peroxide Intermediate—Fig. 1 shows on the left the reaction of catalase and cyanide as recorded spectrophotometrically in the rapid flow apparatus (16). The top tracing represents flow velocity and its downward deflection indicates the moment at which the flow is started. At that time, previously formed catalase cyanide is washed out of the capillary observation tube by the discharge of the syringes and is replaced by thoroughly mixed catalase and cyanide. The flow velocities employed correspond to such short times after mixing that the reaction of catalase and cyanide does not proceed to a measurable extent. Thus a solution of free catalase is flowing rapidly down the observation tube. The spectrophotometer records the abrupt decrease of optical density caused by the replacement of catalase cyanide by free catalase. But when the flow stops, the reaction of catalase and cyanide proceeds, and an increase of optical density equivalent to an upward oscillograph deflection of 105 mm. is measured from Fig. 1. This deflection is termed D_1.

In the right-hand record, the same reaction is repeated, but 10 μM of hydrogen peroxide are mixed with the cyanide solution previous to filling the syringes of the rapid flow apparatus. On initiating the flow catalase is now mixed with both cyanide and hydrogen peroxide. The catalase-hydrogen peroxide complex forms completely at the values of flow velocity used, but causes no deflection of the tracing because the measurement was made at 435 mμ, an isosbestic point between the Soret bands of catalase and catalase hydrogen peroxide (1). Thus a solution of the catalase-hydrogen peroxide complex at its saturation value is flowing rapidly down the observation tube, and, as already mentioned, has the same optical density as free catalase. When the flow stops, the reaction of cyanide with catalase hematins not bound to hydrogen peroxide proceeds with the same velocity as in the left-hand section, but gives an upward deflection of only 75 mm. This deflection is termed D_2. Catalase hydrogen peroxide is unstable and decomposes in about a minute (see Fig. 2 or Chance (1)). As this decomposition occurs, cyanide combines with the freed catalase hematin, as indicated by the slowly rising tracing at the end of the right-hand record of Fig. 1. Finally cyanide binds all three hematins and should give the full deflection of 105 mm. measured in the left-hand record (D_1). That this reaction actually occurs is verified by the deflection of the right-hand record before the flow is started. This deflection was recorded several minutes after a previous identical experiment, after the completion of the decomposition of catalase hydrogen peroxide and the consequent combination of cyanide with all the catalase hematins. The change of optical density from catalase cyanide formed in this way to the catalase-hydrogen peroxide complex (which has the same optical density as free catalase at 435 mμ) is equivalent to an oscillograph deflection of 105 mm. and is identical to the deflection obtained in the left-hand section of Fig. 1. Thus the formation of catalase hydrogen peroxide reduces the deflection from 105 to 75 mm.

If all the hematin groups were bound to peroxide, the deflection would have been reduced to zero (see Fig. 3). If only one of the three hematins were bound to peroxide, the deflection would be reduced by 33 per cent; *i.e.*, from 105 to 70 mm. In fact, these data have been calculated by the formula $1 - (D_2/D_1)100$, which gives the percentage reduction in the amount of catalase cyanide formed from catalase hydrogen peroxide compared with that formed from free catalase.

In Fig. 1, the value of $1 - (D_2/D_1)100$ is 28 per cent. The value expected if hydrogen peroxide occupied only one of the three horse liver catalase hematins is actually 27 per cent, not 33 per cent.[1] Thus the experimental figure and the calculated figure verify that about 1 hydrogen peroxide molecule is bound per catalase molecule.

TABLE I

Variation of $1 - (D_2/D_1)$ *with Cyanide Concentration*

3.4 μM of hematin iron horse liver catalase, 10 μM of hydrogen peroxide; $\lambda = 435$ mμ; 0.01 M phosphate buffer, pH 6.5 (Experiment 82b).

	Initial cyanide, μM							
	0.4	0.8	2.0	4.0	8.0	20	50	200
$1 - (D_2/D_1)100$	44	38	27	30	20	18	8	3

TABLE II

Variation of $1 - (D_2/D_1)$ *with Hydrogen Peroxide Concentration*

3.4 μM of hematin iron horse liver catalase, 4 μM of cyanide, λ 435 mμ; 0.01 M phosphate buffer, pH 6.5 (Experiment 84).

	Initial hydrogen peroxide, μM									
	0	1	2	4	5	20	40	100	400	1000
$1 - (D_2/D_1)100$	0	9	17	19	28	28	27	34	35	34

Experiments over a wide range of cyanide and peroxide concentrations are given in Tables I and II, and the results are expressed again by the factor $1 - (D_2/D_1)100$. In Table I, the values of $1 - (D_2/D_1)$ for low cyanide concentrations are considerably higher than the theory requires, but the error in recording the small concentration of catalase cyanide is large. At 8 μM of cyanide and above, the limitations of Equation 3 are exceeded and a decrease of $1 - (D_2/D_1)$ is seen; here cyanide is competing with hydrogen peroxide for catalase hematin. In accordance with the data of Table I and in agreement with Equation 3, 4 μM of cyanide are chosen for the experiments shown in Table II, and the effect of a variation of hydrogen peroxide concentration is studied. The values of $1 - (D_2/D_1)$ for the larger values of hydrogen peroxide concentration are probably accurate and may be compared with the expected value of 27 per cent for a three-hematin catalase.[1] The average experimental value (34 per cent) indicates that the catalase-hydrogen peroxide compound consists of about 1 molecule of hydrogen peroxide per catalase molecule.

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[1] A calculation shows that the decrease in the amount of cyanide compound to be expected is 27 per cent instead of 33 per cent for a liver catalase with one hematin bound, since appreciably more free cyanide is present in this case (see "Appendix").

Competition between Hydrogen Peroxide and Cyanide for Catalase Hematin—According to the experiments just described, catalase hematins not bound to hydrogen peroxide in the catalase-hydrogen peroxide complex can combine readily with cyanide. The reaction of these free hematins is shown by the records of Fig. 1 and by the data of Table II to be independent of hydrogen peroxide concentration, a non-competitive reaction. These free hematins also participate in the destruction of hydrogen peroxide (3) and, therefore, the inhibition of catalase activity by cyanide ion is non-competitive, as is shown by the data of von Euler and Josephson, Stern, and Keilin and Hartree, in which roughly the same degrees of cyanide inhibition of catalase were obtained in spite of large variations in the initial hydrogen peroxide concentration (for a summary, see Chance (3), Table V).

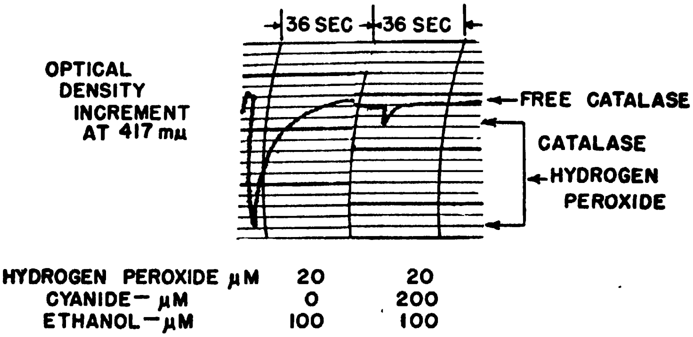

FIG. 2. The effect of cyanide upon the kinetics of the catalase hydrogen peroxide compound measured at the isosbestic point for catalase and catalase cyanide (Experiment 86). 3.4 μM of hematin iron horse liver catalase.

On the other hand, we can now show that the catalase hematin bound to hydrogen peroxide in the catalase-hydrogen peroxide complex is involved in competitive inhibition with cyanide. Table I shows how increasing cyanide concentrations can nearly completely prevent the formation of the catalase-hydrogen peroxide complex. Table II shows how increasing initial hydrogen peroxide concentrations can displace cyanide from the catalase-hydrogen peroxide complex. And finally, Fig. 2 shows directly the decrease in the concentration of the catalase-hydrogen peroxide complex caused by cyanide competing with hydrogen peroxide. This record was obtained at the isosbestic point for catalase and catalase cyanide (417 mμ), and at this wave-length the kinetics of catalase hydrogen peroxide are recorded without interference from the kinetics of catalase cyanide. The left-hand record shows the usual kinetic curve for the rapid formation (sharp drop), and slow disappearance (slow exponential rise) of catalase hydrogen peroxide (see Chance (1)). The right-hand tracing clearly shows that 200 μM of cyanide have "captured" 82 per cent of the catalase hematin bound by hydrogen peroxide in the left-hand record. But the dissociation constant of catalase cyanide (4×10^{-6} M) indicates that nearly 100 per cent of the catalase hematin should have been bound by cyanide if no peroxide were present. The dissociation constant of catalase cyanide calculated from Fig. 2 is $K_I = 44 \times 10^{-6}$ M. Thus hydrogen peroxide and cyanide exhibit competition in this record.

The theoretical increase of the catalase cyanide dissociation constant may be calculated for the usual formula $K_I' = K_I[1 + (x_0/K_m)]$; this is the usual formula for simple competitive inhibition (7), where $x_0 = 20 \times 10^{-6}$ M hydrogen peroxide (initially) and $K_m = 0.6 \times 10^{-6}$ M (see Chance (1); the value of K_m is about half that of the catalase molarity). K_I' is calculated to be 1×10^{-4} M. Thus 65 per cent of the catalase hematin is calculated to be bound by cyanide, and 82 per cent is actually indicated by the right-hand side of Fig. 2.

These simple calculations are in error because the hydrogen peroxide concentration at the time the cyanide ion combines with catalase is less than the initial peroxide concentration, owing to the destruction of peroxide by catalase. This reaction is, of course, partially inhibited by cyanide. Thus the calculated value of K_I' should be considerably less than 1×10^{-4} M and, therefore, in better agreement with the experimental value.

A further proof of the competition between cyanide and peroxide is furnished by the data of Tables I and II, which are further analyzed in Tables III and IV. The data of Table I represent the titration of the catalase-hydrogen peroxide complex with cyanide. Since cyanide ion is competing with the peroxide molecule for the catalase hematin, the dissociation constant is no longer 4×10^{-6} M but is calculated in Table III to be $K_I' = 16 \times 10^{-6}$ M in the range in which the data are most reliable. Thus there is no doubt of competitive inhibition, for the values in Table III are about 3 times greater than the dissociation constant of the catalase cyanide in the absence of peroxide. However, the decomposition of hydrogen peroxide causes less than the amount of competition calculated according to the equation above: $K_I' = 5 \times 10^{-5}$ M.

The data of Table II represent the titration of catalase cyanide with hydrogen peroxide, and the dissociation constant (K') of the peroxide molecule competing with the cyanide ion for the catalase hematin is calculated in Table IV in the range in which the data are most reliable. K' is found to be 2×10^{-6} M compared with the calculated value of 1.2×10^{-6} ($K' = K\,(1 + [\mathrm{CN}]/K_I)$). Here K' is greater than the calculated value. No explanation of this discrepancy can be given at present.

Thus the competition between cyanide and peroxide for catalase hematin involved in the catalase-hydrogen peroxide complex is clearly demonstrated. Nevertheless, the inhibition of the destruction of hydrogen peroxide by catalase is non-competitive, because catalase hematins not involved in this complex are required for the destruction of hydrogen peroxide.

TABLE III

Competition between Cyanide and Hydrogen Peroxide for Catalase Hematin, Calculated from Table I

10 μM of peroxide.

	Initial cyanide concentration, μM			
	8	20	50	200
Measured % catalase hematin bound by cyanide, (33 − (1 − (D_2/D_1) 100)/33)	39	55	76	91
$\left(\frac{e-q}{q}\right)i = \left(\frac{1}{\%}-1\right)i = K'_I$, M × 10^6	12	16	16	20

TABLE IV

Competition between Cyanide and Hydrogen Peroxide for Catalase Hematin Calculated from Table II

4 μM of cyanide.

	Initial hydrogen peroxide concentration, μM				
	1	2	4	5	20
Measured % catalase hematin bound by hydrogen peroxide, (1 − (D_2/D_1) 100)/34	26	50	56	85	85
Calculated equilibrium constant, $\left(\frac{1}{\%}-1\right)[\mathrm{H_2O_2}] = K'$, M × 10^6	2.0	1.4	2.7	0.8	4.0

Composition of Catalase Ethyl Hydrogen Peroxide Compound—Experimental results obtained by use of Method 2 are shown in Fig. 3. Records *A* and *B* are directly comparable with those of Fig. 1. The first reaction (*A*) is simply a calibration of the amount of catalase cyanide that forms from all three hematins. In the second record (*B*), ethyl hydrogen peroxide was first added to catalase in a test-tube, and the mixture was sucked up into the syringe of the rapid flow apparatus and there, 0.3 minute after mixing in the test-tube, was mixed with cyanide in the capillary. This record contrasts sharply with the corresponding record of Fig. 1; no appreciable evidence of the rapid cyanide reaction is obtained, but only of a slow reaction at about the rate at which the catalase-ethyl hydrogen peroxide complex is breaking down. The remaining records confirm this; after 1.5 minutes, an appreciable amount of catalase hematin is free to react rapidly with cyanide, and, after 3 minutes, nearly all the catalase hematin is free. This record is taken to indicate that ethyl hydrogen peroxide can bind all three of the catalase hematins of horse liver. Table V shows the variation of $1 - (D_2/D_1)$ with ethyl hydrogen peroxide concentration. The equilibrium constant for catalase ethyl hydrogen peroxide as calculated from the data of Table V appears to be too large (17), but a considerable portion of the substrate was decomposed before D_2 was measured.

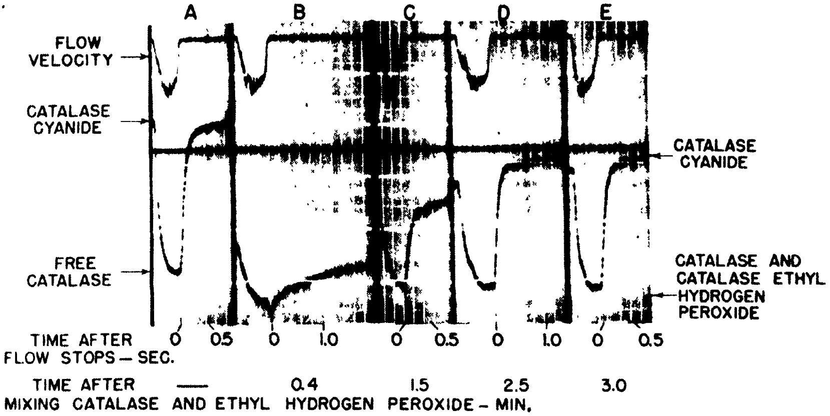

FIG. 3. The kinetics of the formation of catalase cyanide in the absence of ethyl hydrogen peroxide (*A*) and during the decomposition of the catalase-ethyl hydrogen peroxide complex (at 0.4, 1.5, 2.5, and 3 minutes). In record *A*, catalase is mixed with cyanide in the rapid flow apparatus. In records *B* to *E*, the catalase ethyl hydrogen peroxide is preformed and is then mixed with cyanide in the rapid flow apparatus. 3.4 μM of hematin iron horse liver catalase, 10 μM of cyanide, 67 μM of ethyl hydrogen peroxide (Experiment 91d).

The composition of catalase methyl hydrogen peroxide can be assumed to be the same as that of the ethyl hydrogen peroxide complex, since their Soret bands are very similar (18).

Simultaneous Reactions of Hydrogen Peroxide and Ethyl Hydrogen Peroxide with Catalase Hematin—Strong evidence for the accuracy of these conclusions is afforded by using ethyl hydrogen peroxide instead of cyanide in the reaction of catalase and hydrogen peroxide. Since ethyl hydrogen peroxide combines more slowly than cyanide (2, 17), a somewhat greater concentration of the former is required.

These measurements were made at 410 mμ, at which the extinction coefficients of the catalase peroxide compounds are large and, per mole of hematin iron bound, are very nearly equal (18). Thus these data record the sum of the concentrations of catalase hydrogen peroxide and catalase ethyl hydrogen peroxide.

A typical experiment is shown in Fig. 4. In record *A*, the saturation value for catalase ethyl hydrogen peroxide is recorded on a rapid time scale (D_A = 70 mm.) on the bottom line and on a slow time scale directly above. In record *B*, the saturation value (D_B = 45 mm.) and life time of the intermediate compound are decreased by the addition of 400 μM of ethyl alcohol so that the succeeding records can be completed more rapidly (17).

Table V

Effect of Ethyl Hydrogen Peroxide Concentration upon Percentage of Catalase Hematins Free to React with Cyanide

3.4 μM of hematin iron horse liver catalase, 10 μM of cyanide; 0.01 M phosphate buffer, pH 6.5; λ = 435 mμ (Experiment 91d).

	Ethyl hydrogen peroxide, μM			
	17	33	67	130
$1 - (D_2/D_1)100$	48	79	94	100

In record *C*, the saturation value (D_C = 21 mm.) and the kinetics of catalase hydrogen peroxide are recorded. This compound forms very rapidly, nearly completely at the fastest flow velocity. Thus there is only a very small change of its concentration when the flow stops. The corresponding record (above) on a slow time scale shows that alcohol has also accelerated the decomposition of this intermediate (*cf.* Fig. 2, left-hand section).

If now both hydrogen peroxide and ethyl hydrogen peroxide react simultaneously with catalase hematin, as in record *E*, the hydrogen peroxide compound forms first, as indicated by the 21 mm. rapid drop of the oscillograph tracing as the flow starts.[2] After the flow stops, the slower reaction of catalase and ethyl hydrogen peroxide gets under way, but finds one of the catalase hematins already bound to hydrogen peroxide. Thus the extent of the slow reaction (D_E) is only 27 mm. as compared with D_B = 65 mm. in record *B*. As the record above on a slow time scale shows, the reaction proceeds as in record *B*; the hydrogen peroxide compound soon breaks down, and all three hematins are available for ethyl hydrogen peroxide. There is no indication in this slow record of the conversion from the catalase-hydrogen peroxide complex to the catalase-ethyl hydrogen peroxide complex, since the extinction coefficients per hematin are very nearly equal.

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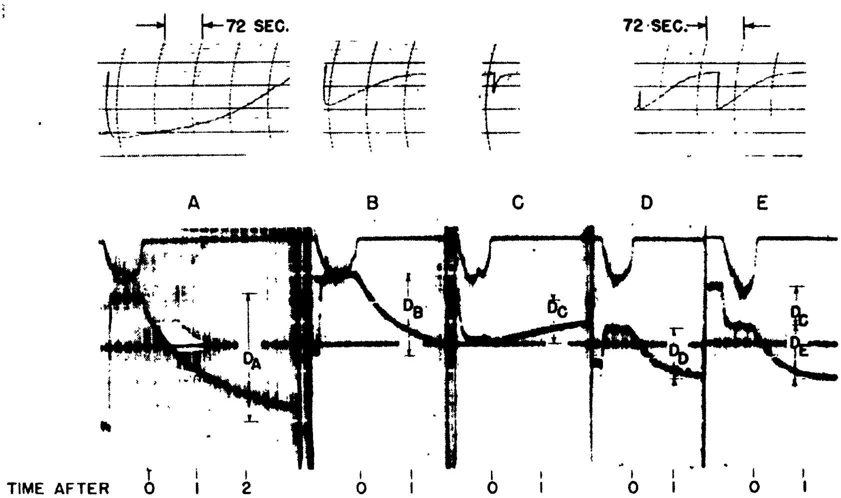
[2] This record depicts what would happen if some hydrogen peroxide were accidentally present in the ethyl hydrogen peroxide. That in excess of the enzyme molarity would be rapidly decomposed and the remainder would react exactly as shown in record *E*. Indications of such a sharp step have not been noticed in the kinetics of formation of ethyl hydrogen peroxide compounds.



	A	B	C	D	E
TIME AFTER FLOW STOPS – SEC.	0 1 2	0 1	0 1	0 1	0 1
ETHYL HYDROGEN PEROXIDE – μM	133	133	0	133	133
ETHYL ALCOHOL – μM	0	400	400	400	400
HYDROGEN PEROXIDE – μM	0	0	40	40	40

FIG. 4. The kinetics of the formation (lower section) and the course of the reaction (upper section) of catalase and ethyl hydrogen peroxide in the absence (*A*, *B*) and in the presence of hydrogen peroxide (*D*, *E*). 3.4 μM of hematin iron horse liver catalase; λ = 410 mμ, pH 6.5, 0.01 M phosphate (Experiment 91a). The optical density changes are as follows: D_A, from free catalase to saturated catalase ethyl hydrogen peroxide; D_B, from free catalase to 0.65 saturated catalase ethyl hydrogen peroxide; D_C, from free catalase to saturated catalase hydrogen peroxide; D_D and D_E, from saturated catalase hydrogen peroxide to 0.65 saturated catalase ethyl hydrogen peroxide.

Record *D*, made just a few seconds after a previous injection, demonstrates the great kinetic difference between the two catalase peroxides. Since the capillary had just been filled with reactants before record *D* was started, the initial base-line corresponds to a mixture of the two catalase peroxides at a little less than their saturation values. Rapid flow of fresh reactants prevents the formation of the ethyl hydrogen peroxide compound but not the hydrogen peroxide compound. Thus the tracing does not rise to the optical density of free catalase, only to that of catalase hydrogen peroxide. After the flow stops, the ethyl hydrogen peroxide reaction proceeds, and the subsequent portions of record *D* duplicate those of record *E*.

A simple calculation gives the number of hematins bound by hydrogen peroxide, since all these can be bound by ethyl hydrogen peroxide.

TABLE VI

Composition of Catalase-Hydrogen Peroxide in Liver Catalase

Hematin compound	Density increment	Ratio of density increments	Expected ratio
	mm.		
$CatH_2O_2$, D_C	21	1.0	1.0
$Cat(C_2H_5OOH)_2$, D_ED_A/D_B	42	2.0	2.0
$Cat(C_2H_5OOH)_3$, D_A	70	3.3	3.0

TABLE VII

Composition of Catalase-Hydrogen Peroxide in Erythrocyte Catalase

Compound	Density increment	Ratio of density increments	Expected ratio
	mm.		
$CatH_2O_2$, D_C	23	1.0	1.0
$Cat(C_2H_5OOH)_3$, D_ED_A/D_B	63	2.7	3.0
$Cat(C_2H_5OOH)_4$, D_A	92	4.0	4.0

The ratio $1 - (D_E/D_B) = 1 - (27/45) = 40$ per cent as compared with the expected value of 33 per cent. The data are summarized in Table VI, and the value of D_E is corrected for partial saturation (D_A/D_B) to give 42 mm.

The extinction coefficients of these compounds per mole of hematin iron are nearly equal (18); the data confirm the conclusions from the cyanide experiments (Table II).

In experiments in which a 1.35 μM horse erythrocyte catalase having four intact hematins was used, the data of Table VII have been obtained by a repetition of Fig. 4. The value of $1 - (D_E/D_B) = 31$ per cent compared with the expected value of 25 per cent. The data are, however, in agreement with those obtained by using the three-hematin liver catalase, and therefore show that the composition of catalase hydrogen peroxide is unrelated to the bile pigment content of the catalase.

Furthermore, no change in the composition of the catalase-hydrogen peroxide complex in a liver or an erythrocyte preparation is indicated by the change in optical density at 410 mμ on formation of the complex: 23 mm. for 1.35 μM of erythrocyte catalase and 20 mm. for 1.17 μM of liver catalase.

Composition of Catalase Hydrogen Peroxide in High Biliverdin Catalase—Bonnichsen has prepared some catalases of high biliverdin content (19). One of these preparations was tested by these methods in order to detect with greater sensitivity the effect of a variation of the number of hematins present in the catalase preparation upon the composition of catalase hydrogen peroxide.

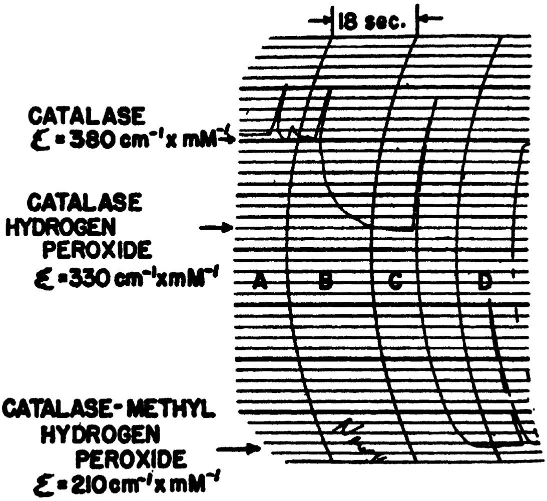


FIG. 5. The composition of catalase hydrogen peroxide formed in the presence of the notatin system. 0.95 μM of horse blood catalase, 0.04 μM of notatin added at *A*, 6.7 μM of glucose added at *B*, 10 μM of methyl hydrogen peroxide added at *C* and at *D*; $\lambda = 405$ mμ, pH 7.0, 0.01 M phosphate buffer (Experiment 251).

This catalase, when compared to a solution of erythrocyte catalase of equal optical density at 405 mμ gives 36 per cent less cyanide compound at 435 mμ and 39 per cent less methyl hydrogen peroxide compound at 405 mμ (18) and may be considered to have about 2.7 hematins. On the basis of a cyanide-hydrogen peroxide test similar to that in Table II, the value of $1 - (D_2/D_1)$ is found to be 38 per cent, and on the basis of a hydrogen peroxide-methyl hydrogen peroxide test according to the method just described, the value of $1 - (D_{[illegible]}/D_{B})$ is 39 per cent.

Thus the composition of the catalase hydrogen peroxide is again shown to be unaffected by a rather large change of bile pigment content, from 0 to 1.3 catalase hematins.

Composition of Catalase Hydrogen Peroxide Formed in Presence of Notatin, Glucose, and Oxygen—The formation of catalase hydrogen peroxide I from hydrogen peroxide produced by the notatin system has been reported (2). Since a continuous supply of hydrogen peroxide is available, the complex is stable for several minutes, and the number of free catalase hematins is simply determined by the addition of excess alkyl hydrogen peroxide, as in Fig. 5.

In these tests, a recording spectrophotometer equipped with a 1.3 cm. cuvette was used. The formation of catalase hydrogen peroxide is shown at *B* and gives a deflection of 8.5 scale divisions on Fig. 5. As soon as a steady state is reached, an excess of methyl hydrogen peroxide is added, and the total density increment is 28.5 scale divisions. Thus 8.5/28.5 or 30 per cent of the four catalase hematins is bound in the hydrogen peroxide complex.

These reactions provide a simple method for determining the number of intact hematins in a catalase containing biliverdin. If the ordinary spectrophotometer is used, about 30 times stronger catalase is required, and some increase in the notatin concentration may be necessary to insure saturation of the catalase-hydrogen peroxide complex. Catalase solutions should be dialyzed thoroughly before this test to remove any alcohol, which would lower the saturation of the catalase-hydrogen peroxide complex owing to the peroxidatic reaction of this complex with ethanol.

DISCUSSION

The preliminary conclusion drawn from previous studies of catalase hydrogen peroxide is substantiated by these studies: the compound consists of very nearly 1 peroxide molecule per catalase molecule. In four determinations by two independent methods, the amount of hydrogen peroxide exceeded 1 per catalase molecule by 26, 21, 23, and 20 per cent (34/27, 40/33, 31/25, and 30/25) for horse erythrocyte and liver catalases. The inherent error of the method would allow a discrepancy of about 12 per cent in these values.[3] The average of these data gives the composition of the catalase hydrogen peroxide as 1.2 ± 0.1 peroxide-bound hematins.

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[3] The error of these methods is usually between 2 and 4 per cent of the total deflection measured. Since the deflection due to the catalase-hydrogen peroxide complex is only one-third to one-fourth of the total deflection, the error is correspondingly greater. These errors are due to inadequate gain of the light intensity control, irregularities in the relative delivery from the syringes, and, in the case of horse liver catalase, errors in the determination of the number of intact hematins.

There are two interpretations of the result. The first is that the catalase hematins are identical in all respects and are completely independent. In this case, each one of the four catalase hematin groups is slightly over 0.25 saturated with hydrogen peroxide. The cause of the partial saturation of the enzyme-substrate complex is readily explained by the "purely kinetic" theory of catalase activity (2); catalase activity involves the consecutive reactions of hydrogen peroxide molecules with catalase and with catalase hydrogen peroxide, and the relative values of the velocity constants of the first and second reactions determine the saturation of the catalase-hydrogen peroxide complex in the steady state (2). This explanation, however, requires modifications to account for the constant composition of the catalase-hydrogen peroxide complex in catalases containing bile pigment. According to this theory, the measured composition of the complex would decrease with a decrease in the number of intact hematin groups; a 2.7 hematin catalase should have only 0.8 peroxide-bound hematin per catalase molecule, in contrast with the experimental data. Explanations for this discrepancy between the theory and the experiments can be obtained by postulating interaction between pairs of catalase hematins (L. Pauling, personal communication). But no evidence of such interaction has yet been found in the reaction of catalase with cyanide (3) or with methyl hydrogen peroxide (17).

The second interpretation is that the composition of the catalase-hydrogen peroxide complex is not a statistical effect but is due to "special properties" of the catalase-hydrogen peroxide complex. After the combination of hydrogen peroxide with any one of the catalase hematins, the catalase molecule acquires "special properties," which permit the rapid destruction of hydrogen peroxide at the free catalase hematins. According to this simple theory, the composition of the catalase-hydrogen peroxide complex would be exactly 1 peroxide molecule per catalase molecule and would be independent of the bile pigment content of the catalase molecule. The latter effect is in accord with these experiments, but the former conflicts with the composition of catalase hydrogen peroxide of 1.2 peroxide molecules per catalase molecule. Also there are no "special properties" of the catalase-alkyl hydrogen peroxide complexes (17).

The composition of the catalase-hydrogen peroxide complex is remarkably independent of the peroxide concentration: no significant change of its spectrum was obtained in 4 mM of hydrogen peroxide (1) or in the presence of the very dilute hydrogen peroxide ($\sim 10^{-9}$ M) generated by notatin, glucose, and oxygen (2), a total range of about 10^6 in hydrogen peroxide concentration.

That the composition of the catalase-hydrogen peroxide complex is a consequence of the "catalatic" reaction (the rapid breakdown of hydrogen peroxide by catalase) is clearly shown by comparison with the composition of the catalase-alkyl hydrogen peroxide complexes in which all the intact catalase hematin groups are bound to alkyl hydrogen peroxide and no "catalatic" reaction is observed; the breakdown of dilute alkyl hydrogen peroxide solutions by catalase in the absence of alcohols is very slow (2).

Since the catalase-hydrogen peroxide complex forms from an erythrocyte catalase having no bile pigment, there is no reason for supposing that, in liver catalase, a combination with the bile pigment hematins is responsible for the catalase-hydrogen peroxide complex.

The non-competitive inhibition of the destruction of hydrogen peroxide by catalase has led some investigators to conclude that cyanide and peroxide do not attach to the same place on the catalase hematin (3, 20). However, non-competitive inhibition is, in this case, clearly due to the combination of cyanide with the free hematins of the catalase-hydrogen peroxide complex. The fact that catalase activity is inhibited by a combination of these hematin groups with cyanide is proof that they are necessary for catalase activity.

In these experiments, it has been possible to demonstrate spectroscopically the competition between cyanide and peroxide for catalase hematin. Thus cyanide and peroxide do combine at the same point on catalase hematin. Therefore the earlier explanations of the non-competitive inhibition of catalase activity are incorrect and are now unnecessary.

SUMMARY

1. In the catalase-hydrogen peroxide complex, 1.2 ± 0.1 hematin groups are occupied by hydrogen peroxide as determined by the reaction of this complex with cyanide or alkyl hydrogen peroxides.

2. The composition of the complex is not appreciably altered by a bile pigment content equivalent to 1.3 hematin groups or by a large variation in the hydrogen peroxide concentration.

3. Hydrogen peroxide is shown to combine with the iron atom of catalase hematin by competition between cyanide and hydrogen peroxide.

4. In the catalase-alkyl hydrogen peroxide complexes, all the intact catalase hematins are bound to peroxide.

5. The non-competitive inhibition of catalase activity by cyanide demonstrates that catalase hematins not involved in the catalase-hydrogen peroxide complex are required for the destruction of hydrogen peroxide.

APPENDIX

Calculation of Change in Amount of Catalase-Cyanide Formed in Presence and Absence of Hydrogen Peroxide—The concentration of free cyanide is greater in the presence of hydrogen peroxide and a correction to the concentrations of catalase cyanide is calculated as follows:

In the absence of hydrogen peroxide and for a liver catalase (E), the combination with cyanide (I) is represented as in an earlier paper (3). The initial enzyme concentration is e_3, and the initial cyanide concentration is i_0. The concentration of catalase cyanide (EI_3) is q_3. Thus the free enzyme concentration is $e_3 - q_3$, and the free cyanide concentration is $i_0 - q_3$.

$$E + 3I \underset{k_6}{\overset{k_5}{\longleftrightarrow}} EI_3 \tag{1}$$

Since the iron atoms of catalase act independently, the equilibrium conditions are equivalent to the reaction of a single cyanide ion with a single iron atom. The dissociation constant is to be calculated; therefore, the equations are written in the reciprocal form.

$$K_I = \frac{(e_3 - q_3)(i_0 - q_3)}{q_3} \tag{2}$$

In the presence of enough hydrogen peroxide to convert E into ES, the catalase-hydrogen peroxide complex, Equation 3 is written, in which the symbols have the same meanings as in Equation 1.

$$ES + 2I \underset{k_6}{\overset{k_5}{\longleftrightarrow}} ESI_2 \tag{3}$$

$$K_I' = \frac{(e_2 - q_2)(i_0 - q_2)}{q_2} \tag{4}$$

Solving Equations 2 and 4 for q_3 and q_2 respectively gives

$$q_3^2 - q_3(i_0 + e_3 + K_I) + e_3 i_0 = 0, \qquad q_3 = \frac{-b - \sqrt{b^2 - 4c}}{2}$$

$$q_2^2 - q_2(i_0 + e_2 + K_I') + e_2 i_0 = 0, \qquad q_2 = \frac{-b' - \sqrt{(b')^2 - 4c'}}{2}$$

Then

$$\frac{q_3}{q_2} = \frac{-b - \sqrt{b^2 - 4c}}{-b' - \sqrt{(b')^2 - 4c'}} \tag{5}$$

where $b = -(i_0 + e_3 + K_I)$, $c = e_3 i_0$; $b' = -(i_0 + e_2 + K_I')$, $c' = e_2 i_0$. It is here assumed that $K_I = K_I'$.

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THE PRIMARY AND SECONDARY COMPOUNDS OF CATALASE AND METHYL OR ETHYL HYDROGEN PEROXIDE

I. SPECTRA*

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In this and following papers, the properties of two new catalytically active enzyme-substrate compounds of catalase are given. Paper I gives spectroscopic data, Paper II and III, data on kinetics and activity, and, in Paper IV, the reactions of these enzyme-substrate compounds with hydrogen peroxide are discussed.

The red catalase ethyl hydrogen peroxide compound studied by Stern (1) is characterized by two sharp bands in the green region of the spectrum and requires about 0.3 M ethyl hydrogen peroxide for maximum spectroscopic effects. Stern's compound is, however, a secondary product of the reaction of enzyme and substrate. The primary reaction product is a green compound which has a single diffuse band in the red region of the spectrum and requires only a very small excess of ethyl hydrogen peroxide for maximum spectroscopic effects ($\sim 5 \times 10^{-5}$ M). Catalase is here shown to form completely analogous primary and secondary compounds with methyl hydrogen peroxide (2). These primary (I) and secondary (II) compounds resemble the green and red peroxidase hydrogen peroxide compounds (see Theorell (3)).

The spectra of the primary compounds of catalase with these alkyl hydrogen peroxides are related to that of the catalase-hydrogen peroxide complex (4) and give an indication of the spectrum of the latter compound in regions for which data have not yet been obtained.

Preparations—Bonnichsen's (5) catalase preparations were used in these experiments, and many thanks are due him. Ethyl hydrogen peroxide[1] was prepared and standardized as described by Stern (1). According to his tests, a pure material gave the same results, and since the concentrations employed here are about 0.001 to 0.0001 of those he employed, the effect of an impurity would be smaller. Methyl hydrogen peroxide was prepared as described by Reiche and Hitz (6). The distillate was about

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* This is Paper 3 of a series on catalases and peroxidases.

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[1] Credit is due Miss Pero Kara for this preparation.

0.3 M. The concentrations were determined by titration as described by Stern (1) but more readily by the spectrophotometric method (see Reiche (7)).

Visual Spectroscopy of Catalase Alkyl Hydrogen Peroxides—Stern (1) found absorption bands at 535.5 and 580 mμ for his compound of catalase and ethyl hydrogen peroxide. In his experiments there was always an appreciable delay in the formation of the red compound; it was preceded by a greenish color which was attributed by him to "concomitant pigments in the enzyme solutions." A repetition of Stern's visual tests with pure catalase and either methyl or ethyl hydrogen peroxide shows that a diffuse absorption band appears at 670 mμ and is responsible for the transient green color of the catalase alkyl hydrogen peroxide solutions. Using a 5 to 10 μM catalase solution in a 10 cm. tube, one sees the band at 670 mμ very clearly for about 4 seconds upon the addition of 40 μM of methyl hydrogen peroxide and less distinctly for about 20 seconds upon the addition of 160 μM of ethyl hydrogen peroxide. At the end of these times, the band at 670 mμ disappears, and that at 630 mμ reappears. With stronger catalase, the band at 670 mμ appears to extend from 640 mμ towards 700 mμ. On addition of larger amounts of the alkyl hydrogen peroxides, the green compounds are converted into the red compounds, which are stable for several minutes. The red methyl and ethyl hydrogen peroxide compounds of catalase have the same visible absorption spectrum, judged from the hand spectroscope, and their bands lie at 536 and 572 mμ, at approximately the positions found previously by Stern.

Stern's red compound is therefore a secondary compound which is preceded by a primary green compound, as in the case of the red and green peroxidase-hydrogen peroxide compounds (3).

Relation between Spectral Shifts in Visible and in Soret Region—The photosensitivity and the changes of extinction coefficient are so small at 670 and 580 mμ that kinetic studies with the rapid flow apparatus are uneconomical of enzyme solution, and use of the Soret region is always preferable. Therefore a series of experiments has been carried out in a 1.33 cm. cuvette to show the correspondence between the spectral shifts in the visible and Soret regions. The capillary cuvette of the flow apparatus (8) is replaced by a pair of 1.33 cm. cuvettes. One cuvette is filled with 1.13 μM of catalase solution (5 cc.), and upon addition of 0.05 cc. of 0.3 M ethyl hydrogen peroxide and stirring, the transmission changes are recorded directly, as shown in Fig. 1. The constant transmission of the catalase solution is followed by a sharp spike, indicating the moment at which the ethyl hydrogen peroxide is stirred in. Then the kinetics of the intermediate compound are recorded. The sensitivity is adjusted appropriately for each record. In records *B* and *D*, the transmission changes are larger than a few per cent and are not, therefore, directly proportional to the concentration of the intermediate compound. In record *A* at 650 mμ, the green compound rapidly forms directly from catalase, and in a few minutes disappears. At 580 mμ there is very little, if any, evidence of a rapid reaction; only the slow formation of the relatively stable red compound at about the same rate as the green compound disappeared at 650 mμ. Thus it is shown that the green compound, in accordance with the visual tests, forms first and is slowly converted into the red compound. Similar records were obtained at 423 and 435 mμ. At the wave-lengths 650 and 423 mμ, there are isosbestic points (wave-lengths of equal extinction coefficient) for catalase and compound II, and at 580 and 435 mμ, there are isosbestic points for catalase and compound I. Thus the kinetics of these two compounds can be independently recorded at these points. The kinetics of compound I are usually recorded at 405 mμ, because small concentrations of substrate give negligible amounts of compound II before compound I decomposes into free catalase (9).

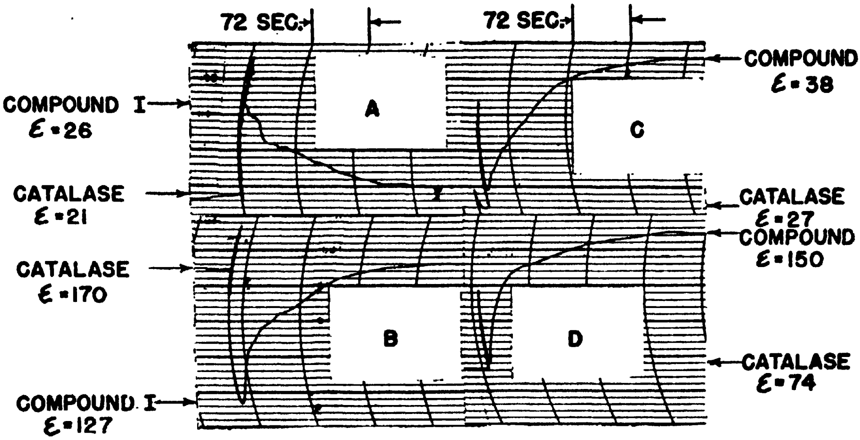

FIG. 1. The relationship between the spectral shifts in the formation of catalase ethyl hydrogen peroxide I and II in the visible region and in the region of the Soret band of catalase. The units of ϵ are cm.$^{-1}$ × mM^{-1}. The wave-lengths and sensitivities (amperes per scale division) used in the four records are as follows: record *A*, 650 mμ, 6×10^{-12}; *B*, 423 mμ, 6.2×10^{-10}; *C*, 580 mμ, 1.3×10^{-10}; *D*, 435 mμ, 7.3×10^{-10}. The spacing between two of the heavier lines represents 10 scale divisions. An open 1.33 cm. cuvette was used in these experiments. The spike indicates the moment at which the ethyl hydrogen peroxide was stirred into the solution. The final ethyl hydrogen peroxide concentration was 3 mM. 1.13 μM of horse liver catalase, pH 7.0, 0.01 M phosphate (Experiment 145d).

Soret Bands of Primary and Secondary Compounds—As the data of Fig. 1 show, the secondary compounds are stable for several minutes when dilute (~1 μM) catalase solutions are used, and the Soret band can be measured directly in the Beckman spectrophotometer, as shown in Fig. 2, Curves IIa and IIb. To insure that compound I has been completely converted into compound II, the spectrum is measured after the initial change of optical density at 421 mμ upon addition of the peroxide has decreased to zero. Also the density at 435 mμ is checked before and after the measurement to insure that complex II has not decomposed.

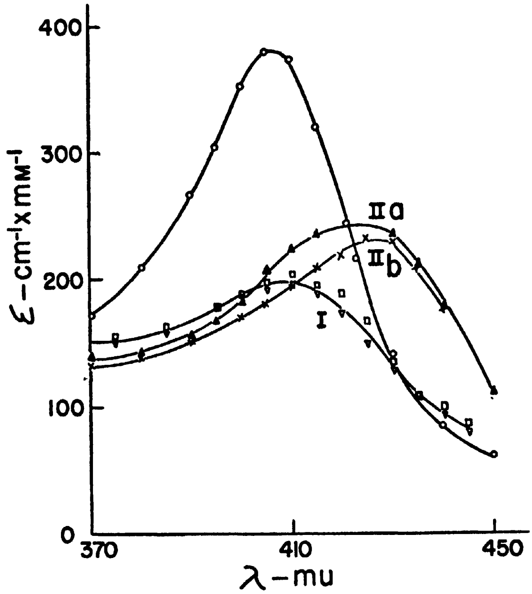

FIG. 2. The Soret bands of the primary and secondary catalase-alkyl hydrogen peroxide complexes. The curve following the circles is the Soret band of catalase. Curves IIa and IIb are the Soret bands of catalase methyl hydrogen peroxide II and catalase ethyl hydrogen peroxide II respectively. The Soret band of the primary catalase-alkyl hydrogen peroxide complex is given in Curve I and is obtained by subtracting from Curve IIa the changes of extinction coefficient found in the rapid flow apparatus corresponding to the conversion of compound I to II. Curve IIa, 0.64 μM horse erythrocyte catalase, 300 μM of methyl hydrogen peroxide (Experiment 139). Curve IIb, 0.57 μM of catalase, 5, 10, 100 mM of ethyl hydrogen peroxide. Curve I (△) 0.66 μM of horse erythrocyte catalase, 100 μM of methyl hydrogen peroxide; and (□) 200 μM of ethyl hydrogen peroxide (Experiments 127b and 130a). All curves, pH 6.5, 0.01 M phosphate buffer.

It has been found with both horse radish peroxidase and lactoperoxidase (10) that the extinction coefficient of the secondary complexes is the same, regardless of whether methyl or ethyl hydrogen peroxide is used. Here the shapes of Curves IIa and IIb are similar, but the extinction coefficient of catalase ethyl hydrogen peroxide II as measured in the

Beckman spectrophotometer is usually found to be slightly less (ϵ_{425} = 232 cm.$^{-1}$ × mM^{-1}) than that of the methyl hydrogen peroxide complex (ϵ_{425} = 242 cm.$^{-1}$ × mM^{-1}). Since 10 to 100 times more ethyl hydrogen peroxide than methyl hydrogen peroxide are required to stabilize the ethyl hydrogen peroxide complex for the duration of the experiment, some catalase is probably destroyed when ethyl hydrogen peroxide is used. The data for methyl hydrogen peroxide are therefore considered to be more accurate.

In the ordinary spectrophotometer, the conversion of complex I into complex II begins before satisfactory measurements can be made (see Fig. 1). Therefore, the rapid flow apparatus is used to measure the change of optical density from complex I to complex II as illustrated by Figs. 3 and 8 of Chance (9). The values obtained in such experiments are identical within the experimental error for the methyl and ethyl hydrogen peroxide complexes, as Fig. 2 shows. These changes of optical density are converted into changes of extinction coefficient as before (4), except that here the optical density change at 435 mμ (where complex I and catalase have isosbestic points) is used to calculate the scale factor for the conversion from change of optical density to change of extinction coefficient at the other wave-lengths. By subtracting these changes of extinction coefficient from Curve IIa, Curve I is obtained.

If Curve I is constructed by applying the changes of extinction coefficient from catalase to complex I to the catalase spectrum, the curve lies above that given in Fig. 2 in the region of 405 mμ. The method described above is considered to be more accurate because the changes of extinction coefficient between Curves II and I are smaller and the shapes of the curves are more alike than are the catalase spectrum and Curve I. Therefore, errors caused by excessive spectral interval have a small effect upon the values of extinction coefficient of Curve I.

In experiments with horse liver catalase, the Soret bands of the secondary complexes of methyl and ethyl hydrogen peroxide were found to be nearly identical. The maximum extinction coefficient (ϵ_{425} = 204 cm.$^{-1}$ × mM^{-1}) is less than that obtained with horse erythrocyte catalase in Fig. 2 (ϵ_{425} = 242 cm.$^{-1}$ × mM^{-1}) in accordance with studies of the cyanide complex (11).

DISCUSSION

The primary catalase-alkyl hydrogen peroxide complexes are now identified as greenish colored complexes which precede the red secondary complexes, the general effect closely resembling that found by Theorell for horse radish peroxidase and hydrogen peroxide (3). As observed in the hand spectroscope, the addition of alkyl hydrogen peroxide to catalase gives a large decrease of absorption in the green region and the formation of a diffuse band at 670 mμ. A similar band of the primary catalase-hydrogen peroxide complex has been demonstrated spectrophotometrically (2).

The Soret bands of the primary complexes of catalase with methyl or ethyl hydrogen peroxide are the same but are much less intense than the Soret band of catalase hydrogen peroxide. The Soret bands of the primary peroxidase-peroxide complexes are identical, regardless of whether hydrogen peroxide or alkyl hydrogen peroxide is attached to peroxidase hematin. It is reasonable to conclude that the decrease of extinction coefficient of the Soret bands of the catalase-peroxide complexes per hematin iron group bound to peroxide is identical for hydrogen peroxides and alkyl hydrogen peroxides. Thus the difference between the intensity of the Soret bands of the primary catalase-hydrogen peroxide and catalase-alkyl hydrogen peroxide complexes is caused by a difference in the number of hematins bound to peroxide.

The Soret bands of the primary complexes of catalase and methyl or ethyl hydrogen peroxide are about half the intensity of the Soret band of the free enzyme and are of a shape generally similar to that of the free enzyme. No other known compound of catalase has a similar Soret band. In fact, a Soret band of this type is obtained only when the porphyrin ring is opened, as in the degradation of hemoglobin (12) or when the bile pigment content of catalase is increased (11), for example, by treatment with a large excess of hydrogen peroxide (13). The decrease of extinction coefficient at 405 mμ obtained on formation of the primary alkyl hydrogen peroxide complex ($\Delta\epsilon_{405} \doteq 45$ cm.$^{-1}$ × mM^{-1} per hematin iron bound to peroxide) roughly equals the difference between a three- and four-hematin catalase ($\Delta\epsilon_{405} = 380$ to $340 = 40$ cm.$^{-1}$ × mM^{-1} for one hematin converted to bile pigment). But the peroxides combine directly with the iron atom of catalase and not to the methine bridges of the porphyrin ring (14). Speculation as to whether the porphyrin ring is actually oxidized on formation of the primary complex by electron transfer from the iron-peroxide complex and is then reduced on reaction with the reducing substrate or acceptor affords very interesting possibilities but is premature at this time.

The visible absorption bands of the secondary catalase-alkyl hydrogen peroxide complexes lie at 536 and 572 mμ, at approximately the positions found by Stern (1). Recently the secondary catalase-hydrogen peroxide complex has been found, and it has the same visible absorption bands. Whereas the Soret bands of the peroxidase-alkyl hydrogen peroxide complexes are identical (10), the extinction coefficient of catalase ethyl hydrogen peroxide II was found to be slightly less than that of catalase methyl hydrogen peroxide II. This is probably caused by slight destruction of the enzyme by the large excess of ethyl hydrogen peroxide.

The valence of the iron in the primary catalase-alkyl hydrogen peroxide complexes is established as ferric because of the lack of any carbon monoxide inhibition of the oxidation of alcohols (9). Also, cyanide has been shown to compete with peroxide for the iron atoms of catalase hematins (14).

The secondary catalase-peroxide complexes are not enzymatically active, and their valence is not indicated by these activity tests. However, the relationships between absorption spectra and iron bonding, which were established by Theorell (15) and recently summarized by Hartree (16), are applicable here. Catalase cyanide has been shown to be a ferric iron compound with covalent bonds by magnetic susceptibility tests (17). Its visible and Soret bands (compare Figs. 2 and 3) resemble those of the secondary catalase-peroxide complexes. By analogy, these secondary catalase-peroxide complexes are also ferric compounds with covalent bonds.

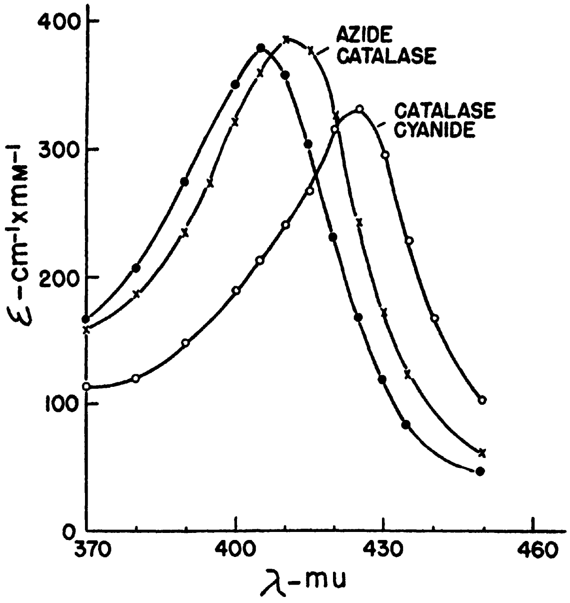

FIG. 3. The Soret bands of catalase cyanide and azide catalase. ● represents the Soret band of catalase. 0.6 μM of horse blood catalase, 70 μM of cyanide, or 3 mM of azide; pH 6.5, 0.01 M phosphate buffer (Experiments 285 and 284).

No measurements have yet been made of the magnetic susceptibility of the primary catalase-peroxide complexes, and spectral analogies are weak because there is no compound of catalase which has a similar spectrum.

Azide catalase is colored green and has been found to be a ferric compound with ionic bonds (17). But there is a very remote similarity between the Soret bands of azide catalase and the primary catalase-peroxide complexes, as Figs. 2 and 3 clearly show. Only by analogy with the primary peroxidase-peroxide complexes can the bond type of the primary catalase-peroxide complexes be established as ionic. The experimental data for the bond type of the primary peroxidase-peroxide complexes is, however, weak (10).

The formation of a covalent iron-peroxide bond does not necessarily explain the slow conversion of the primary to the secondary catalase-peroxide complexes; such a shift can occur in peroxidase as rapidly as peroxide combines with the iron atom to form the primary complex (10). Nor is it true that covalent compounds of hematins form very slowly; the covalent catalase (18) and peroxidase (19) cyanides form fairly rapidly.

Evidence is now accumulating for the importance and generality of the green primary peroxide complexes of presumably ionic bonds in catalysis by catalase, horseradish peroxidase (10), and lactoperoxidase (10). On the other hand, it is not yet established that cytochrome *c* peroxidase forms such a complex (20), but the ability of the horseradish peroxidase to oxidize ferrocytochrome *c* (10) indicates that the reaction mechanism is very similar in these two cases. Verdoperoxidase has been observed to form a green complex but no red complex (21). No green complex of methemoglobin with peroxides has yet been observed.

SUMMARY

1. Catalase forms primary and secondary enzyme-substrate compounds with methyl or ethyl hydrogen peroxide.

2. The primary complexes are green and have a diffuse absorption band starting at 670 mμ. The secondary complexes are red and have visible bands at 536 and 572 mμ in approximately the positions found by Stern in his work on catalase ethyl hydrogen peroxide II.

3. These complexes contain ferric iron according to the lack of carbon monoxide inhibition and spectral analogy. In the secondary complexes, the iron is probably bound by covalent bonds according to spectral analogy with the covalent cyanide compound. In the primary complexes, the iron is probably bound by ionic bonds according to spectral analogy with the primary peroxidase-peroxide complexes.

4. In the primary catalase-alkyl hydrogen peroxide complexes, the Soret bands are similar in shape to that of the free enzyme but are shifted towards the visible region of the spectrum by several millimicrons. At 405 mμ, the decrease of extinction coefficient is the same for ethyl and methyl hydrogen peroxide, about 180 $\text{cm.}^{-1} \times \text{mM}^{-1}$ for an erythrocyte

catalase or about 45 cm.$^{-1}$ × mM^{-1} per hematin iron group bound to peroxide.

5. The Soret band of the secondary catalase-methyl hydrogen peroxide complex resembles that of catalase cyanide and has a maximum value, ϵ_{422} = 242 cm.$^{-1}$ × mM^{-1}. The Soret band of the secondary catalase-ethyl hydrogen peroxide complex was found to be slightly smaller, owing probably to catalase destruction.

6. It is probable that the reaction of all catalases and peroxidases with peroxides involves the primary formation of the same type of complex. The exact nature of this complex is not known; the spectroscopic data suggest that changes in the porphyrin ring of the hematin group may be involved.

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THE PRIMARY AND SECONDARY COMPOUNDS OF CATALASE AND METHYL OR ETHYL HYDROGEN PEROXIDE

II. KINETICS AND ACTIVITY*

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The catalase-ethyl hydrogen peroxide compound studied by Stern (1) formed slowly, even in the presence of a very large concentration of ethyl hydrogen peroxide. In the previous paper (2), Stern's compound has been shown to be preceded by a green primary compound. These kinetic studies show that the primary compound forms rapidly, has a high affinity[1] for its substrate, and quantitatively fulfils the requirements for a Michaelis intermediate. The primary compound of catalase and methyl hydrogen peroxide has similar properties.

Keilin and Hartree have demonstrated the oxidation of ethanol to acetaldehyde by catalase and ethyl hydrogen peroxide (3). Whereas they believed that this peroxidatic oxidation was caused by the production of hydrogen peroxide from ethyl hydrogen peroxide, the reaction is shown here to be a peroxidatic reaction of the primary catalase ethyl hydrogen peroxide compound with ethanol. The oxidation of acceptors[2] such as the lower alcohols is a property common to the primary catalase alkyl hydrogen peroxides and catalase hydrogen peroxide (4). The reactivity of these three compounds towards alcohols is about the same.

The variation of the velocity of the reaction of catalase with alkyl hydrogen peroxide and with alcohols of different sizes indicates that the accessibility of the iron atom in catalase hematin is limited to small substrate and acceptor molecules.

The relative reactivity of the primary and secondary enzyme-substrate compounds towards alcohols has been evaluated and the inhibition of the oxidation of alcohols by the formation of the secondary catalase alkyl hydrogen peroxide compounds is demonstrated.

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* This is Paper 4 of a series on catalases and peroxidases.

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[1] "Affinity" is frequently used here to mean the reciprocal of the dissociation constant of the enzyme-substrate compound.

[2] "Acceptor" is here defined as the molecule which is oxidized by the enzyme-substrate complex.

Kinetics and Equilibria of Primary and Secondary Compounds of Catalase with Ethyl or Methyl Hydrogen Peroxide—The reactions studied in this section are the combination of catalase (E) with alkyl hydrogen peroxide (S) to form the primary complexes (ES_I),

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES_I \tag{1}$$

and their conversion into the secondary complexes (ES_{II})

$$ES_I \underset{k_8}{\overset{k_7}{\rightleftarrows}} ES_{II} \tag{2}$$

The iron atoms of catalase are assumed to react independently.

Catalase Ethyl Hydrogen Peroxide I—In dilute ethyl hydrogen peroxide and catalase solutions, the formation of compound II is so slow that the kinetics of compound I are satisfactorily recorded at 400 to 410 mμ before a considerable quantity of compound II has formed. Fig. 1 shows the formation and disappearance of compound I in the presence of several different initial ethyl hydrogen peroxide concentrations. These records were obtained in the rapid flow apparatus. The capillary cuvette is initially filled with the end-product of the previous experiment, free catalase, and then is rapidly refilled with mixed but unchanged catalase and ethyl hydrogen peroxide. Their reaction proceeds and causes an abrupt decrease in optical density, as indicated by the downward deflection of the recorder tracings in Fig. 1. A steady state ensues for about 30 seconds and then the intermediate compound disappears, owing to exhaustion of the substrate. The kinetics of catalase ethyl hydrogen peroxide I bear a strong resemblance to those of peroxidase hydrogen peroxide II in the presence of ascorbic acid (5).

The initial phases of the reactions of Fig. 1 have been recorded on a faster time scale and, at a particular initial concentration of ethyl hydrogen peroxide, the course of the reaction is shown in the tracing and graph of Fig. 2, *a* and *b*, to follow the second order equation. As in previous records with the rapid flow apparatus, the downward deflection of the top tracing of the kinetic record represents the flow velocity of the reactants down the observation tube. Since this is a slow reaction, the optical density of free catalase is obtained while the reactants are flowing. When the flow stops, the combination of catalase and ethyl hydrogen peroxide proceeds, and the kinetics of formation are recorded directly against time. The half times of the formation of the complex are recorded in Table I for various initial ethyl hydrogen peroxide concentrations and are seen to be in fairly good accord with the second order equation. Thus compound I is formed by the bimolecular combination of all the catalase hematins

(6) with ethyl hydrogen peroxide and $k_1 = 2 \times 10^4 \text{ M}^{-1} \times \text{sec.}^{-1}$. The dissociation constant for catalase and ethyl hydrogen peroxide is here calculated without regard for the disappearance of ethyl hydrogen peroxide due to the enzymatic activity and is found to be 5.3×10^{-6} M. The correction for the loss of substrate is given later.

Catalase Methyl Hydrogen Peroxide I—The primary catalase methyl hydrogen peroxide compound forms more rapidly than does the ethyl hydrogen peroxide compound, as is shown by comparing the tracings *A* and *B* of Fig. 2, *a*. This reaction is clearly of the second order as shown by Graph B of Fig. 2, *b*, and over the range covered by the data of Table II, the average value of k_1 is $0.85 \times 10^6 \text{ M}^{-1} \times \text{sec.}^{-1}$. The larger velocity constant for the formation of this compound gives a nearly proportional increase in the affinity of catalase for methyl hydrogen peroxide compared with ethyl hydrogen peroxide. Since the enzyme concentration is larger than the dissociation constant, the error of this determination is rather large ($K \approx 2 \times 10^{-7}$ M). The effect of enzymatic decomposition of

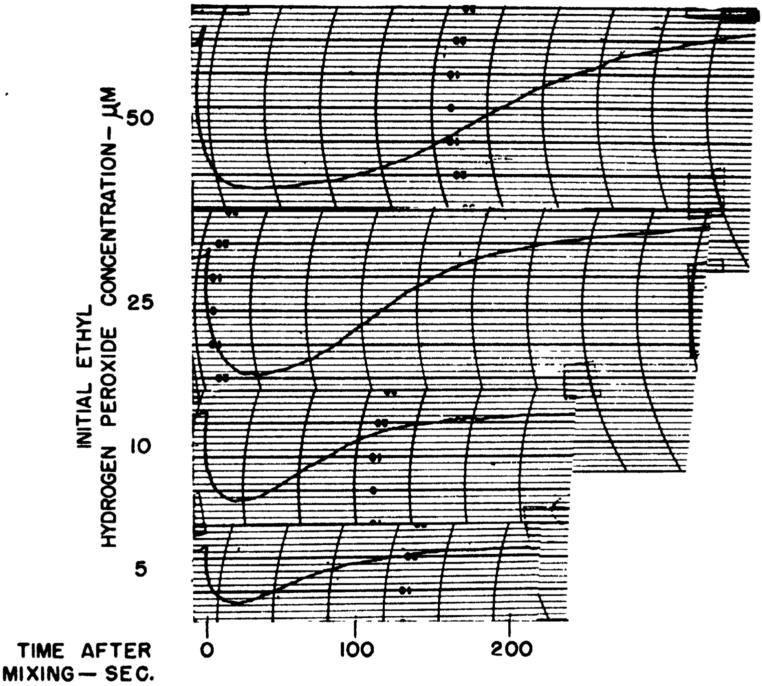

FIG. 1. Rapid spectrophotometric recordings of the formation and disappearance of catalase ethyl hydrogen peroxide I. The saturation value corresponds to 48 scale divisions. 5.7 μM of hematin iron horse liver catalase; λ = 405 mμ, pH 6.7, 0.01 M phosphate (Experiment 77).

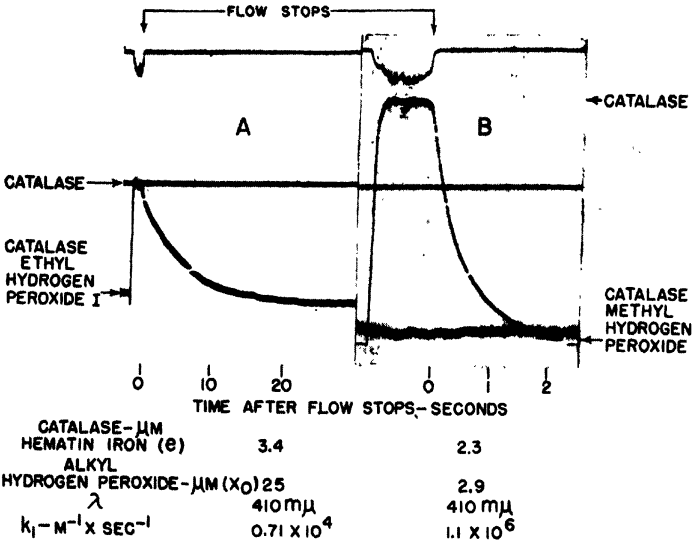

FIG. 2, *a*

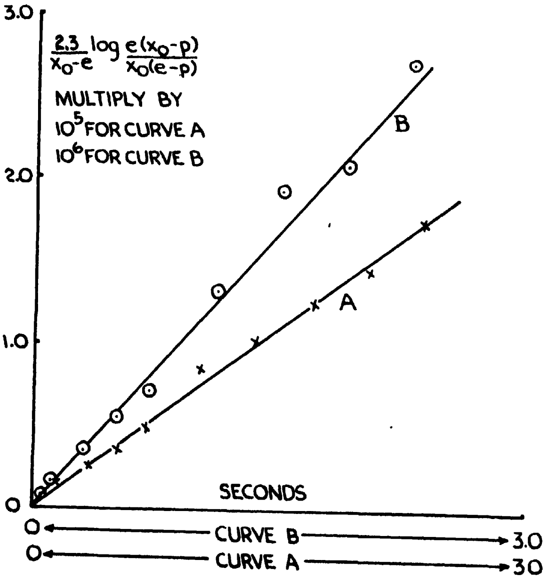

FIG. 2, *b*

FIG. 2. (*a*) Records of the formation of catalase ethyl hydrogen peroxide I (*A*) and catalase methyl hydrogen peroxide I (*B*) obtained by the stopped flow method. (*b*) These curves are plotted according to the second order equation in Graphs A and B respectively. The sensitivity of the apparatus was about twice as great in tracing *B* as in *A*. The rises of the tracings of the original records have been retouched.

the substrate has again been neglected and will be discussed later, as will the method of calculation of the data in Table II on the velocity constant for the decomposition of the intermediate compound.

Catalase Ethyl Hydrogen Peroxide II—The relative rates of formation of the primary and secondary compounds of catalase and ethyl hydrogen peroxide are shown by the records of Fig. 3. At 395 mμ, compound I is seen to be partially formed at the highest value of flow velocity corresponding to a time after mixing of several milliseconds and the reaction is complete about 0.1 second after the flow stops. At 435 mμ, the formation of compound II does not begin until the formation of compound I is nearly

TABLE I

Kinetics and Equilibrium of Primary Catalase-Ethyl Hydrogen Peroxide Complex

Horse liver catalase; pH 6.7, 0.01 M phosphate, λ = 410 mμ (Experiments 77 and 91e).

Initial ethyl hydrogen peroxide concentration, μM	5	10	25	50	25	100	200
Initial concentration of catalase hematin iron, μM	5.5	5.5	5.5	5.5	3.4	3.4	3.4
Maximum concentration of complex I, μM	2.0	3.1	5.3	5.5	2.8	3.3	3.4
Half time for formation of complex I, *sec.*	5.3	2.6	1.6	0.9	3.0	0.70	0.36
2nd order velocity constant, $k_1 \times 10^{-4}$ $M^{-1} \times sec.^{-1}$	3.4	3.0	1.8	1.7	1.0	1.0	0.90
Dissociation constant, $K \times 10^{8}$ M	5.2	5.3			5.3		

TABLE II

Velocity Constants for Formation and Spontaneous Decomposition of Primary Catalase-Methyl Hydrogen Peroxide Complex

1.9 μM of hematin iron guinea pig liver catalase; pH 6.7, 0.01 M phosphate, λ = 405 mμ (Experiment 120b).

Initial methyl hydrogen peroxide, μM	0.29	0.58	1.5	2.9	15	58
Concentration of complex I at *t*, μM		0.24	0.60	0.95	0.95	0.62
t, *sec.*		0.33	0.39	0.30	0.06	0.0086
2nd order velocity constant, $k_1 \times 10^{-6}$ $M^{-1} \times sec.^{-1}$		0.90	0.84	0.96	0.76	0.80
Dissociation constant, $K \times 10^{7}$ M		3	2			
Maximum concentration of complex I, μM	0.40	0.48	1.20	1.9	1.9	1.9
1st order velocity constant for decomposition of catalase methyl hydrogen peroxide (by Equation 12), $sec.^{-1}$	0.017		0.016	0.015		

complete. The wave-length, 395 mμ, is approximately the isosbestic point (wave-length of equal extinction coefficient) for compounds I and II, and this record shows a slight decrease of optical density due to the conversion of compound I to compound II.

Although compound II does not form until after compound I, the rate of formation of compound II does increase with increasing ethyl hydrogen peroxide concentration, as is shown in Table III. The deviations from a second order reaction over the range of ethyl hydrogen peroxide concentrations are very great.

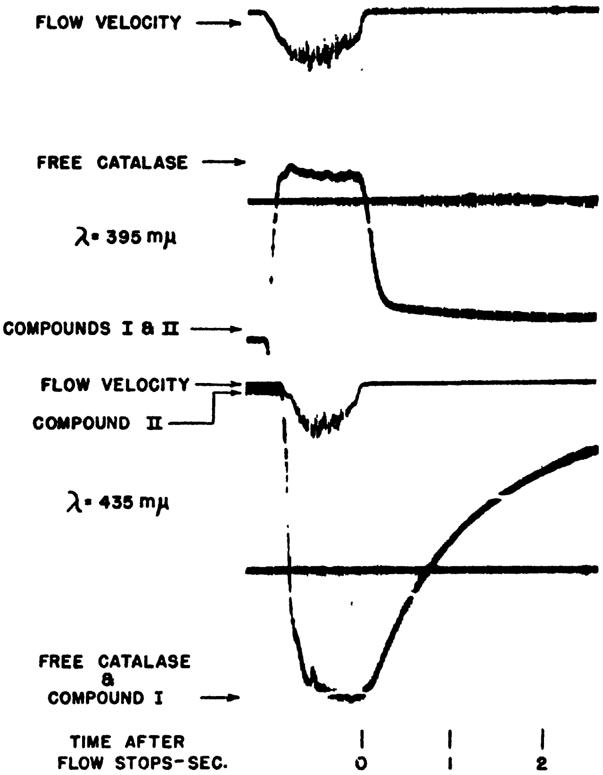

FIG. 3. A comparison of the reaction kinetics of compounds I and II of guinea pig liver catalase (3.6 μM of hematin iron) and ethyl hydrogen peroxide (50 mM) at two wave-lengths (Experiment 126).

The slowness of this reaction had already been recognized by Stern (1) and was discussed as the "lag period" of a duration of about 3.8 seconds. Later experiments of Stern and DuBois (7), as estimated from their Fig. 8, give a half time of 2 seconds and a value of $k_1 = 3.5\ \text{M}^{-1} \times \text{sec.}^{-1}$ in 0.1 M ethyl hydrogen peroxide. This value agrees roughly with an extrapolation of Table III.

But the great discrepancy between these data and those of Stern (1) lies in the dissociation constant for compound II. While Stern required 0.3 M ethyl hydrogen peroxide to achieve saturation of compound II at 3°, the dissociation constant of compound II given in Table III is about 2 × 10^{-5} M. Neither Stern's value nor this value is, however, corrected for decomposition of the ethyl hydrogen peroxide during the formation of compound II.

TABLE III

Kinetics and Equilibrium of Secondary Catalase-Ethyl Hydrogen Peroxide Complex

3.6 μM of hematin iron guinea pig liver catalase; pH 6.7, 0.01 M phosphate, λ = 435 mμ (Experiments 126 and 147).

Initial concentration of ethyl hydrogen peroxide, *μM*	20	40	60	4000	10,000	50,000
Maximum concentration of complex II, *μM*	2.0	2.5	2.4	3.6	3.6	3.6
Half time for formation of complex II, *sec.*	57	81	47	17	6.7	1.0
2nd order velocity constant, M^{-1} × *sec.*$^{-1}$	610	215	240	10	10.3	14
Dissociation constant, *M* × 10^5	1.3	1.6	3.0			

TABLE IV

Kinetics and Equilibrium of Secondary Catalase-Methyl Hydrogen Peroxide Complex

2.4 μM of hematin iron horse liver catalase; pH 6.7, 0.01 M phosphate, λ = 435 mμ (Experiment 136).

Initial methyl hydrogen peroxide concentration, *μM*	3.3	16	33	50	91	330
Maximum concentration of complex II, *μM*	1.1	1.5	2.2	2.3	2.4	2.4
Half time for formation of complex II, *sec.*	84	53	27	35	17	9.5
2nd order velocity constant, M^{-1} × *sec.*$^{-1}$	1250	840	830	410	460	220
Dissociation constant, *M* × 10^6	2.6	8.7	2.8	2.2		

Catalase Methyl Hydrogen Peroxide II—As in the case of the primary compounds, catalase methyl hydrogen peroxide II forms more rapidly and has a smaller dissociation constant than the ethyl hydrogen peroxide compound. Records are shown later (Fig. 8) which indicate a similar delay in the formation of compound II until compound I has formed. Nevertheless, the data of Table IV show that the kinetics of this reaction follow a second order equation better than do those of catalase ethyl hydrogen peroxide II and definitely suggest that the formation of compound II involves more than a first order conversion of compound I to compound II. The equilibrium constant ($K = 4 \times 10^{-6}$ M) is later recalculated.

Decomposition of Primary Compounds

General Theory—The decomposition of catalase hydrogen peroxide into free catalase has been shown to occur in two ways: first, in a slow spontaneous reaction, and second, in a fast reaction in which the alcohol is oxidized to aldehyde (4). Fig. 1 shows that the primary catalase-ethyl hydrogen peroxide complex spontaneously decomposes into free catalase in the absence of alcohols and later data will show the rapid reaction of this complex with alcohols. Since the method of calculation of the velocity constants for the decomposition of the intermediate compound from kinetic data is similar in both cases, the formulas for these two conditions will be derived.

Whereas the reaction kinetics of catalase hydrogen peroxide represent the decomposition of but 1 molecule of catalase hydrogen peroxide (because the "catalatic" reaction[3] destroys all the peroxide except that which becomes attached to one of the catalase hematins (4)), there is no measurable catalatic activity with the alkyl hydrogen peroxide, and the reaction kinetics represent the successive recombinations of catalase (E) with alkyl hydrogen peroxide (S) to form the primary complex (ES) and to decompose spontaneously or to react with the acceptor (A).[4] The concentrations of the reactants at any time are written under the symbols E, A, S, and ES. The nature of the products Q_1 and Q_2 and the effect of the secondary complexes upon the activity are discussed later.

$$\underset{(e-p)}{E} + \underset{(x)}{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(p)}{ES_{\text{I}}} \qquad (3)$$

$$\underset{(p)}{ES_{\text{I}}} \xrightarrow{k_3} E + Q_1 \qquad (4)$$

$$\underset{(p)}{ES_{\text{I}}} + \underset{(a)}{A} \xrightarrow{k_4} E + Q_2 \qquad (5)$$

The differential equations for the formation of the intermediate compound

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[3] "Catalatic" activity is here defined as the reaction of a hemoprotein with hydrogen peroxide, giving water and oxygen.

[4] The kinetics are similar to those already treated previously (5) for peroxidase and hydrogen peroxide. In this case, however, full account is taken of the acceptor concentration and the equations are thereby more complex.

(dp/dt) and the disappearance of alkyl hydrogen peroxide $(-\,dx/dt)$ and of alcohol $(-\,da/dt)$ are

$$\frac{dp}{dt} = k_1 x(e - p) - k_2 p - k_3 p - k_4 a p \tag{6}$$

$$-\frac{dx}{dt} = k_1 x(e - p) - k_3 p \tag{7}$$

$$-\frac{da}{dt} = k_4 a p \tag{8}$$

By addition of Equations 4 and 5

$$\frac{dp}{dt} + \frac{dx}{dt} = -(k_3 + k_4\, a)p \tag{9}$$

and at any time

$$x = x_0 - p - (k_3 + k_4 a_0) \int_0^t p dt \tag{10}$$

where x_0 is the initial value of x and a is assumed to be constant at its initial value a_0. On integrating between $t = 0$ and $t = \infty$ and substituting limits at $t = 0$ of $p = 0$, $x = x_0$, and at $t = \infty$, $p = 0$ and $x = 0$,

$$k_3' = k_3 + k_4 a_0 = \frac{x_0}{\int_0^\infty p dt} \tag{11}$$

From recent solutions of Equations 6, 7, and 8 obtained by the differential analyser[5] the factor $\int_0^\infty p dt$ is very nearly equal to $p_{\mathrm{max.}} . t_{\frac{1}{2}\,\mathrm{off}}$ where $t_{\frac{1}{2}\,\mathrm{off}}$ is the time interval from $t = 0$ until p has fallen from its maximum value $p_{\mathrm{max.}}$ to $p_{\mathrm{max.}}/2$. This had previously been found to be the case for the simpler differential equations (5).

$$k_3' = k_3 + k_4 a_0 = \frac{x_0}{p_{\mathrm{max.}}\, t_{\frac{1}{2}\,\mathrm{off}}} \tag{12}$$

It is thus possible to evaluate k_3 by setting $a_0 = 0$. The value of k_3 thus found is applied as a correction to the rate when $a_0 \neq 0$, and in this way the true value of k_4 is found. The quantity $p_{\mathrm{max.}}$ must be evaluated in moles per liter. In these experiments (see Figs. 1, 4, and 5), $p_{\mathrm{max.}}$ is usually obtained as a fraction of the maximum value, $p = e$. For the peroxidatic reactions of the catalase alkyl hydrogen peroxidase in which all the intact hematins may be bound, e is the enzyme concentration in terms of hematin iron. The value of e may be determined directly as pyridine hemochromogen with the expenditure of catalase solution or more conveniently from the optical density of catalase at 405 mμ. The extinction coefficients of horse blood and liver catalases are known, and in these experiments the hematin iron content is obtained by multiplying the catalase molarity by 4 or 3 respectively. For low hematin catalases, it is preferable to use the more direct pyridine hemochromogen test (8).

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[5] Massachusetts Institute of Technology Center of Analysis, Differential Analyser Problem 164. These solutions, as those used previously (5), were obtained for 1 μM of enzyme only. The empirical relations, however, appear to be valid over a reasonable range of enzyme concentrations.

In the reactions of catalase hydrogen peroxide, the value $p = e$ is approximately the catalase molarity, since only about one catalase hematin is bound to hydrogen peroxide. Nevertheless, the values of k_4 calculated for hydrogen peroxide or for the alkyl hydrogen peroxides are directly comparable, since they refer to the reaction velocity of one hematin peroxide group with the acceptor.

TABLE V

Calculation of k_3 from Data of Fig. 1 by Equations 11 and 12

$[C_2H_5OH]$, μM (x_0)	5	10	25	50
$\int_0^\infty pdt$, μM × sec.	130	250	510	1060
k_3 from Equation 11, sec.$^{-1}$	0.040	0.040	0.049	0.047
$p_{max.}$, μM	2.0	3.1	5.3	5.5
$t_{\frac{1}{2}\ off}$, sec.	65	85	130	200
k_3 from Equation 12, sec.$^{-1}$	0.039	0.038	0.036	0.044

Breakdown Constant and Michaelis Constant for Catalase Ethyl Hydrogen Peroxide I—A check of Equations 11 and 12 is afforded by the data of Table V, and it is seen that there is good agreement between the values of k_3 calculated from the data of Fig. 1. The constancy of k_3 over the 10-fold range of ethyl hydrogen peroxide concentration gives strong support to the mechanism represented by Equations 3 and 4; a single intermediate compound determines the reaction velocity, as was found in the case of peroxidase, hydrogen peroxide, and ascorbic acid or leucomalachite green (5). This decomposition of catalase ethyl hydrogen peroxide is, however, a "spontaneous" reaction, since no acceptor was added.

It is possible that some alcohol was present in the ethyl hydrogen peroxide owing to its decomposition after distillation, and it is necessary to prove that the "spontaneous" decomposition actually occurs. If alcohol were present, the values of k_3 found in Table V should have been calculated according to Equations 11 or 12 in this manner:

$$k_3' = k_3 + k_4 f x_0 \tag{13}$$

where f is the fraction of x_0 which is ethanol. It will be shown later that k_4 is about 2000 $\text{M}^{-1} \times \text{sec.}^{-1}$. The value of f can be determined in two ways. (1) If $k_3 = 0$ and alcohol is responsible for the observed value of k_3, what is the required alcohol concentration? From the relation $k_3' = k_4 f x_0$, f must range from a minimum of 0.46 at $x_0 = 50$ μM to a maximum of 3.5 at $x_0 = 5$ μM. It is very unlikely that the alcohol concentration derived from decomposition of ethyl hydrogen peroxide could be so high. (2) If k_3 is not zero, then the portion of the activity carried by the alcohol can be calculated from the increase of k_3' with x_0,

$$\frac{dk_3'}{dx_0} = k_4 f \tag{14}$$

Between $x_0 = 5$ to $x_0 = 50$ μM, f can be no higher than 0.08. Thus the contribution to the observed rate by alcohol is negligible (0 to 0.008 sec.^{-1}).

The constancy of k_3 in Table V indicates that the mechanism of decomposition of the catalase ethyl hydrogen peroxide compound in Fig. 1 is due to a spontaneous decomposition of the enzyme-substrate compound as found with peroxidase and the concentration of alcohol which might lead to a peroxidatic reaction is too small to be a significant factor in these reaction kinetics. At larger values of x_0 the effect of an alcohol impurity is shown to be quite pronounced (9).

Michaelis Constant—On the assumption that k_2, the reversible breakdown of the catalase-ethyl hydrogen peroxide complex, is negligible, and under the condition that no acceptor is present ($a_0 = 0$) the Michaelis constant is simply the ratio of the rates of decomposition and formation of the intermediate compound, approximately 2×10^{-6} M.

The Michaelis constant may also be calculated directly from the curves of Fig. 1 in a manner described previously (5). The method of calculation used in Tables I and II is not exact, since the value of x when $p = p_{\text{max.}}$ must be calculated from Equation 10 by using the mean value of k_3 in Table V. Then K_m is readily calculated according to the steady state formula

$$K_m = x\left(\frac{e}{p_{\text{max.}}} - 1\right) \tag{15}$$

The values of K_m are calculated for 5 and 10 μM of ethyl hydrogen peroxide only, since at higher concentrations a small error in $p_{\text{max.}}$ gives a large change in the expression $(e/p_{\text{max.}}) - 1$. The average value calculated in Table VI ($K_m = 3 \times 10^{-6}$ M) is somewhat higher than that calculated from kinetic data. A possible explanation of this discrepancy is that k_2, the reversible breakdown of the intermediate compound, is not zero but has a value of 0.02 sec.^{-1}.

Breakdown Constant and Michaelis Constant for Catalase Methyl Hydrogen Peroxide I—The data given in Table II have been calculated by Equation 12 and show that the spontaneous breakdown of catalase methyl hydrogen peroxide I is somewhat slower (0.016 sec.$^{-1}$) but otherwise quite similar to that of catalase ethyl hydrogen peroxide I. The constancy of k_3 lends support to the mechanism of Equations 3 and 4.

From kinetic data, the Michaelis constant for catalase and methyl hydrogen peroxide to form the primary compound is given by the ratio $k_3/k_1 = 0.016/8.5 \times 10^5 = 2 \times 10^{-8}$ M, an enzyme-substrate affinity comparable with that of peroxidase for hydrogen peroxide. The dissociation constant given in Table II was in error, owing to the decomposition of substrate during the formation of the intermediate compound. In a calculation similar to that in Table VI, but on the basis of much less extensive data, the dissociation constant for catalase methyl hydrogen peroxide I is found to be 1×10^{-7} M. Since $k_3 = 2 \times 10^{-8}$ M, k_2 may have a value of about 0.1 sec.$^{-1}$. However, both the values of the dissociation constant and k_2 are maximum values.

TABLE VI

Calculation of K_m from data of Fig. 1 According to Equations 10 and 15

x_0	$\int_0^{t=t \text{ of } p_{\max.}} p\,dt$	$k_3\int_0^{t=t \text{ of } p_{\max.}} p\,dt$	$p_{\max.}$	x	$\frac{e}{p_{\max.}} - 1$	K_m
μM	μM × sec.	μM	μM	μM		μM
5	34	1.4	2.0	1.6	1.8	2.9
10	60	2.4	3.0	4.6	0.85	3.4

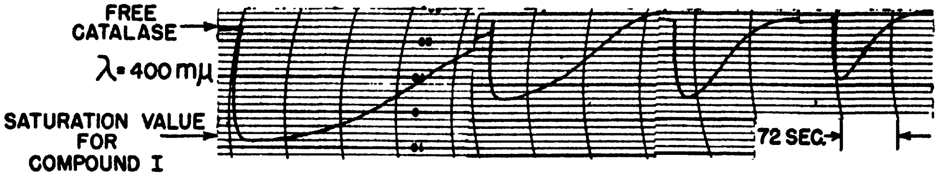
Peroxidatic Activity of Primary Compounds

Catalase Ethyl Hydrogen Peroxide I—If ethanol and ethyl hydrogen peroxide are mixed with catalase, the striking changes of the kinetics of compound I shown in Fig. 4 are obtained. Both $p_{\max.}$ and $t_{\frac{1}{2}\text{ off}}$ decrease regularly with the increase of ethanol concentration. Also the values of k_3' calculated according to Equation 12 are seen to increase regularly. Since $k_3 = 0.04$ sec.$^{-1}$, the values of k_4 are calculated from Equation 12, since a_0 is known and is assumed to be constant throughout the reaction.

If the experiment of Fig. 1 is now repeated in the presence of ethanol, as in Fig. 5, it is seen that the life time of the intermediate compound is much shorter, and the initial concentrations of ethyl hydrogen peroxide required to saturate the enzyme are much larger. The values of k_4 calculated by Equation 12 are relatively constant in spite of the change of saturation of the intermediate compound from 20 to 80 per cent.

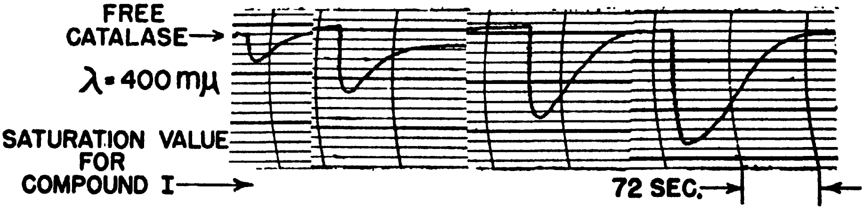
Catalase Methyl Hydrogen Peroxide I—The data of Table VII clearly show that the activity of this intermediate compound towards ethanol is very similar to that of catalase hydrogen peroxide (4) and somewhat less than that of catalase ethyl hydrogen peroxide.

The spectroscopic cycles of catalase methyl hydrogen peroxide I are completed much more rapidly than those with ethyl hydrogen peroxide and are more suitable for detailed experiments. Equation 12 has therefore been tested over a wide range of variation of $p_{\max.}$ in order to obtain evidence for any difference between the four erythrocyte catalase hematins. The constancy of k_4 at both 405 and 421 mμ given by Fig. 6 affords no indication of any difference in the peroxidatic activity of the catalase hematins.



ETHANOL—μM	0	40	100	200
k'_3 – SEC^{-1}	0.04	0.10	0.20	0.42
k_4 – M^{-1} × SEC^{-1} × 10^{-3}	—	1.5	2.0	2.1

FIG. 4. The effect of variation of ethanol concentration upon the kinetics of the catalase ethyl hydrogen peroxide compound. 33 μM of ethyl hydrogen peroxide, 3.4 μM of hematin iron horse liver catalase; pH 6.5, 0.01 phosphate buffer (Experiment 88).



ETHYL HYDROGEN PEROXIDE—μM	6.7	17	33	67
k'_3 – SEC^{-1}	0.53	0.53	0.43	0.40
k_4 – M^{-1} × SEC^{-1} × 10^{-3}	2.5	2.5	2.0	1.8

FIG. 5. The effect of a variation of initial ethyl hydrogen peroxide concentration upon the kinetics of the catalase ethyl hydrogen peroxide compound, 200 μM of ethanol, 3.4 μM of hematin iron horse liver catalase; pH 6.5, 0.01 M phosphate buffer (Experiment 88).

TABLE VII

Velocity Constant for Reaction of Primary Catalase-Methyl Hydrogen Peroxide Complex and Ethanol Calculated by Equation 12

3.3 μM of hematin iron guinea pig liver catalase; pH 6.7, 0.01 M phosphate, λ = 405 mμ (Experiment 123).

Initial methyl hydrogen peroxide concentration, *μM*	5.8	5.8	5.8	5.8
Initial ethyl alcohol concentration (a_0), *μM*	0	200	400	1000
Maximum concentration of complex I, *μM*	3.3	3.1	2.9	2.3
$t_{\frac{1}{2}\,\text{off}}$, *sec.*	59	6.2	4.7	2.4
k_3, k_3', *sec.*$^{-1}$	0.03	0.30	0.43	1.1
k_4a_0, *sec.*$^{-1}$		0.27	0.40	1.1
k_4, M^{-1} × *sec.*$^{-1}$		1350	1000	1100

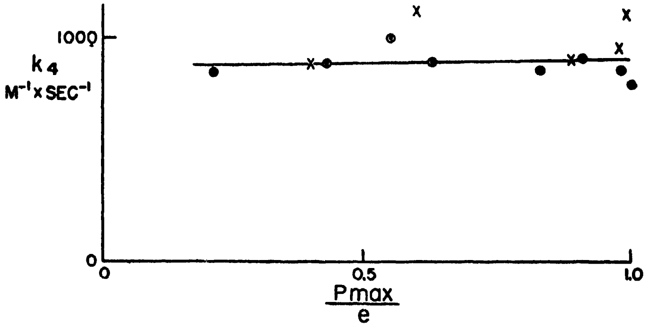


FIG. 6. The relation between p_{max}/e and k_4 at 405 (O) and 421 (X) mμ. 2.36 μM of hematin iron horse blood catalase, 1 mM of ethanol, and methyl hydrogen peroxide concentrations ranging from 1 to 40 μM; pH 6.5, 0.01 M phosphate (Experiment 156a).

TABLE VIII

Comparison of Peroxidatic Activities of Horse Blood and Horse Liver Catalases

21 μM of methyl hydrogen peroxide, 1 mM of ethanol, pH 6.5, 0.01 M phosphate (Experiment 157a). The extinction coefficients and the number of hematins are based upon the data of Bonnichsen (8). $p_{\text{max.}} = 0.95e$.

Type of catalase	ϵ_{405}	No. of hematins	Concentration of hematin iron	$t_{\frac{1}{2}\,\text{off}}$	k_3'	k_3	k_4
	cm.$^{-1}$ × *mM*$^{-1}$		*μM*	*sec.*	*sec.*$^{-1}$	*sec.*$^{-1}$	M^{-1} × *sec.*$^{-1}$
Horse blood..........	380	4	2.36	10.4	0.90	0.01	890
" liver...........	340	3	2.40	10.2	0.90	0.02	880

Table VIII shows that the activities of the intact hematins of liver and blood catalases are very nearly equal.

Further tests have been made of a number of highly purified low hematin catalases prepared by Bonnichsen (8). In these studies, independent methods of estimating the concentration and the number of intact hematins are somewhat involved, and therefore we have evaluated the product of $p_{max.}$ in recorder scale divisions (proportional to the concentration of complex I) and $t_{\frac{1}{2}\,off}$ for constant x_0 and a_0 (see Equation 12). This product is nearly constant in spite of the fact that the amounts of low hematin catalases (as measured by the optical density at 405 mμ required to give equal values of $p_{max.}$) were 2 or more times the amount of horse blood catalase. This indicates that the presence of bile pigment does not reduce the peroxidatic activity of the intact hematin groups.

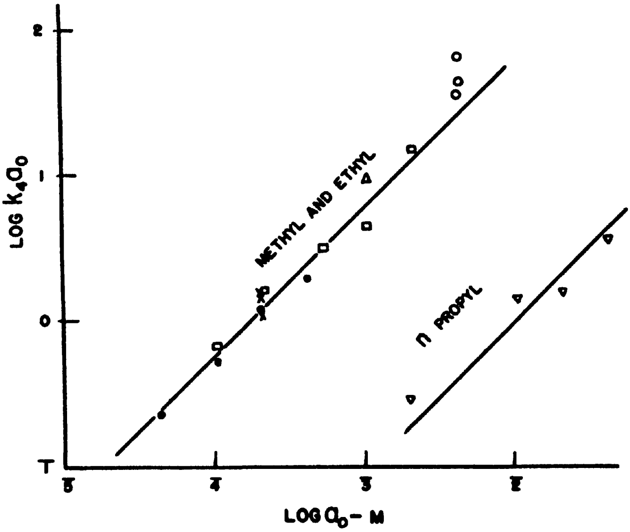


FIG. 7. The constancy of k_4 with alcohol concentration and the relative peroxidatic effect of the catalase ethyl hydrogen peroxide compound upon various alcohols. For ethanol and methanol, $k_4 = 2.1 \times 10^3$; for *n*-propanol, $k_4 = 33\ \text{M}^{-1} \times \text{sec.}^{-1}$. 3.4 μM of hematin iron horse liver catalase. The ethyl hydrogen peroxide concentrations, ● = 33, × = 7, 17, 67, ○ = 330, 670, 1300, △ = 330, and ▽ = 67 μM; □ = 67 μM and represents the methanol data. λ = 400 mμ, pH 6.5, 0.01 M phosphate buffer, temperature 25° (Experiment 88).

A variation of the number of hematins involved in peroxidatic activity by partial saturation of the enzyme with substrate or by conversion of some of the hematins to biliverdin causes no effect upon the reactivity of the remainder.

Comparison of Effect of Various Alcohols—Fig. 7 shows a comparison of the effect of methyl, ethyl, and *n*-propyl alcohol upon k_4a_0 for the ethyl hydrogen peroxide complex. The small scatter of the points from the straight lines shows that the mechanism of Equations 3, 4, and 5 is valid for the various alcohols over the experimental range of concentrations. The data for ethanol also include several different values of ethyl hydrogen peroxide concentration. The velocity constants are about twice as large as those for catalase hydrogen peroxide (4). However, their relative activities are identical; ethyl and methyl alcohols have the same rates and the decrease from them to *n*-propyl alcohol is 59- and 64-fold respectively.

Table IX

Velocity Constants for Reactions of Primary Catalase-Methyl Hydrogen Peroxide Complex I with Methanol and n-Propanol (Calculated by Equation 12)

3.6 μM of hematin iron guinea pig liver catalase; $k_3 = 0.012$ sec.$^{-1}$, pH 6.7, 0.01 M phosphate, $\lambda = 405$ mμ (Experiment 125).

Initial methyl hydrogen peroxide, μM	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Alcohol	Methanol					*n*-Propanol		
Concentration (a_0), μM	100	200	400	800	1600	4000	10,000	20,000
Maximum concentration of complex I, I, μM	3.4	3.2	2.6	2.0	1.4	3.3	3.1	2.9
$t_{\frac{1}{2}}$ off, sec.	6.9	4.7	3.3	1.9	1.5	11	6.7	4.4
k_3', sec.$^{-1}$	0.123	0.192	0.0338	0.762	1.38	0.080	0.140	0.227
k_4a_0, sec.$^{-1}$	0.111	0.180	0.316	0.750	1.37	0.068	0.128	0.215
k_4, M^{-1} × sec.$^{-1}$	1110	900	790	940	860	17	13	11

The methyl hydrogen peroxide complex has a corresponding activity towards methanol and *n*-propanol, as the data of Table IX show. The value of the velocity constants is very nearly equal to those of the catalase-hydrogen peroxide complex in the presence of these alcohols.

The catalase-peroxide complexes have been found to react in a similar fashion with formaldehyde and formate, and details of these reactions will be published later.

Effect of Carbon Monoxide on Peroxidatic Activity—No extensive tests of cyanide inhibition have yet been made, but the spectroscopic data already given (6) permit the prediction that the cyanide effect will be a normal competitive inhibition. The effect of carbon monoxide upon the primary compounds is of especial interest, since an indication of the valence of the hematin iron is given. Table X shows that there is no formation of ferrocatalase carbon monoxide and no inhibition of the activity. There is a slight acceleration of the reaction velocity.

This acceleration of the reaction velocity is probably due to the formation of formic acid by hydration of carbon monoxide. The formate produced in this manner may react with catalase-methyl hydrogen peroxide complex (see above). The data of Table XI support and amplify this explanation. The value of k_3 is increased somewhat when carbon monoxide is bubbled through the substrate only. This may be due to formic acid entrained in the carbon monoxide vapor, to formic acid formed from carbon monoxide, or to the formation of some formic acid upon mixing carbon monoxide with catalase in the capillary of the flow apparatus. In

TABLE X

Effect of Carbon Monoxide upon Reaction of Primary Catalase-Methyl Hydrogen Peroxide Complex and Ethanol

2.36 μM of horse blood catalase, 24 μM of methyl hydrogen peroxide, 1 mM of ethanol; pH 6.5, 0.01 M phosphate, λ = 405 mμ (Experiment 159a).

Maximum concentration of complex I, *μM*	2.1	2.36
Carbon monoxide, *μM*	0	~1000
$t_{\frac{1}{2}\,\mathrm{off}}$, *sec.*$^{-1}$	14.2	10.3
k_3', *sec.*$^{-1}$	0.80	0.98
k_4a_0, *sec.*$^{-1}$	0.74	0.92
k_4, *M*$^{-1}$ × *sec.*$^{-1}$	740	920

TABLE XI

Effect of Carbon Monoxide upon Decomposition of Primary Catalase-Methyl Hydrogen Peroxide Complex

2.36 μM of hematin iron horse blood catalase, 4.4 μM of methyl hydrogen peroxide; pH 6.5, 0.01 M phosphate, λ = 405 mμ (Experiment 160). Solutions bubbled with CO for 5 minutes.

Conditions	Maximum concentration of complex I	$t_{\frac{1}{2}\,\mathrm{off}}$	k_3'	Calculated value of a_0
	μM	*sec.*	*sec.*$^{-1}$	*μM*
(*a*) No CO	2.36	190	0.010	0
(*b*) CO bubbled through methyl hydrogen peroxide solution only	2.10	107	0.018	8
(*c*) CO bubbled through both catalase and methyl hydrogen peroxide solutions	2.2	15.2	0.131	120

any case, the dramatic increase of k_3 after bubbling carbon monoxide through catalase can only be due to the formation of much more acceptor than formed in the absence of catalase. When a value of $k_4 = 1000$ M^{-1} × sec.$^{-1}$ is used for the acceptor formed, its molar concentrations are listed as a_0. These are believed to be the concentrations of formate present in the solutions. In condition (*c*), the final concentration of formate is about 12 per cent of the carbon monoxide concentration.

Inhibition of Peroxidatic Activity by Formation of Secondary Compound—The spectroscopic cycles presented up to this point represent conditions in which a negligible amount of compound II is formed. In order to increase the formation of compound II and to test the activity of mixtures of compounds I and II, a slightly different technique was used. The rapid flow apparatus was replaced by an open 1.33 cm. cuvette so that repeated additions of substrate to the same very dilute enzyme solution could be made.

The general relations between the kinetics of compounds I and II are given by the recordings of Fig. 8, obtained at both 400 and 435 mμ by a repetition of the experiment. At *a* and *a′*, 100 μM of ethanol were added. The momentary density change is due to stirring the solution. At *b* and *b′* and *c* and *c′*, 1.65 μM additions of methyl hydrogen peroxide were made.

At 400 mμ compound I is seen to form and to decompose in the presence of ethanol. At the end of the reaction, however, some of the enzyme has been converted into compound II and does not decompose. The formation of this amount of compound II is recorded at 435 mμ. The delay in the formation of compound II on the first addition of methyl hydrogen peroxide is clearly shown, as is the accumulation of compound II during the kinetics of compound I.

The data of Table XII summarize a series of such experiments in which successive additions of methyl hydrogen peroxide were made, causing the concentration of compound II at the end of the cycles of compound I to increase from 17 to 82 per cent. The activity of that amount of compound I which formed during each successive cycle was evaluated by Equation 12 and was found to be nearly constant, in spite of the presence of the various amounts of compound II.

These data show that (1) the enzymatic activity is inhibited by the formation of compound II; (2) the contribution of compound II to the total activity is less than the experimental error; compound II is inactive; (3) the activity of the portion of the catalase not bound as compound II is not reduced by the presence of a large amount of compound II.

Properties of Secondary Complexes—Table XIII shows data on the slow formation and decomposition of the secondary complexes in dilute catalase solutions. These kinetics were measured in an open cuvette, as in the experiments recorded in Table XII. Here a wave-length of 435 mμ was used to record only the concentration of complex II. In the absence of ethanol, single additions of peroxide to such dilute catalase solutions gave saturation values of complex II. After a steady state period, these complexes decompose, releasing free catalase. If the velocity constants for the formation of the complexes are calculated according to the second order equation (which is not strictly justifiable, as previously shown), maximum values of the dissociation constants (k_8/k_7) are calculated. These maximum values are of the same order of magnitude as the dissociation constants of the primary complexes, and the true values are probably about equal. This indicates that complex I can be nearly quantitatively converted into complex II in the absence of an acceptor, and under these conditions marked inhibition of catalase activity will be obtained.

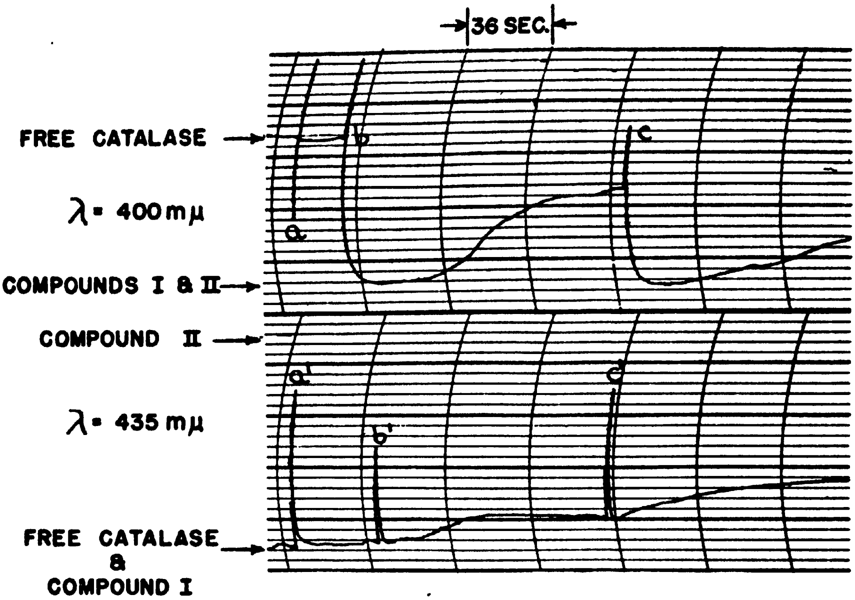


FIG. 8. Comparison of the kinetics of catalase methyl hydrogen peroxides I and II at 400 and 435 mμ. These two separate experiments were carried out in an open cuvette 1.33 cm. in depth. 100 μM of ethanol are added at *a* and *a'* and 1.65 μM of methyl hydrogen peroxide at *b* and *b'* and at *c* and *c'*. The sharp spike indicates the moment of mixing. 0.35 μM of hematin iron horse liver catalase (Experiment 142).

The formation of complex II was repeated and then ethanol was added. While the decomposition of the secondary complexes is accelerated, the reaction velocity of complex II with ethanol computed as k_4 is about 100,000 times smaller than that of complex I; in fact, the accelerated decomposition of complex II in the presence of ethanol is probably due to the reaction of ethanol with a small amount of complex I in equilibrium with complex II, and complex II has negligible peroxidatic activity (see Chance (9)).

TABLE XII

Activity of Primary Catalase-Methyl Hydrogen Peroxide Complex in Presence of Varying Amounts of Secondary Catalase-Methyl Hydrogen Peroxide Complex

200 μM of ethanol, 0.35 μM of hematin iron horse liver catalase; pH 6.7, 0.01 M phosphate, λ = 400 mμ, 1.33 cm. cuvettes (Experiment 142a).

Concentration of methyl hydrogen peroxide added, μM	1.65	1.65	1.65	1.65	1.65	1.65	3.30	1.65
Maximum concentration of complex I, μM	0.20	0.20	0.18	0.17	0.15	0.17	0.10	0.078
Concentration of complex II at completion of cycle of compound I, μM	0.058	0.088	0.13	0.16	0.18	0.21	0.25	0.27
Concentration of complex I converted to complex II during cycle of complex I, μM	0.058	0.029	0.039	0.029	0.024	0.034	0.039	0.015
Maximum % of enzyme concentration in form of complex I during cycle	60	60	55	52	45	36	30	24
% enzyme concentration in form of complex II after completion of cycle of complex I	17	27	39	48	54	64	75	82
$t_{\frac{1}{2}\,\mathrm{off}}$ for cycle of complex I, *sec.*	44	61	54	54	57	59	139	92
k_4, $M^{-1} \times sec.^{-1}$	940	680	850	900	960	1200	1200	1200

Effect of pH upon Peroxidatic Activity of Catalase—The initial activity of catalase in the destruction of hydrogen peroxide is not decreased in acid solutions up to pH 3.5 (10), and Table XIV shows that the peroxi-

TABLE XIII

Kinetics of Formation and Disappearance of Secondary Alkyl-Hydrogen Peroxide Complexes in Dilute Catalase Solutions

λ = 435 mμ, pH 7.0, 0.01 M phosphate buffer (Experiment 148).

Concentration of catalase hematin iron, *μM*	0.35	0.53
Concentration of alkyl hydrogen peroxide added, *μM*	Methyl hydrogen peroxide, 1.65	Ethyl hydrogen peroxide, 15.6
Half time for formation of complex II, *sec.*	1330	420
Reaction velocity constant for formation (k_7) calculated as 2nd order reaction, $M^{-1} \times sec.^{-1}$	320	110
Half time for decomposition of complex II, *sec.*	1.8×10^4	3×10^3
Reaction velocity constant for decomposition (k_8) calculated as 1st order reaction, $sec.^{-1}$	5×10^{-5}	2.3×10^{-4}
Dissociation constant of complex II (k_8/k_7), *M*	0.12×10^{-6}	2.0×10^{-6}
Half time for decomposition of complex II in presence of ethanol, *sec.*	460	80
Concentration of ethanol added, *mM*	400	170
Reaction velocity constant for decomposition in presence of alcohol divided by alcohol concentration, k_4, $M^{-1} \times sec.^{-1}$	3.8×10^{-3}	5.3×10^{-3}

TABLE XIV

Effect of pH upon Activity of Catalase Methyl Hydrogen Peroxide with Ethanol

0.46 μM of hematin iron horse blood catalase, 16 μM of methyl hydrogen peroxide, 2 *mM* of ethanol (Experiment 294b).

pH Buffer, *M*	3.65 0.001 (Phosphate)	7.0 0.001 (Phosphate)	8 8 0.07 (Borate)	> 11 (NaOH)
Maximum concentration of complex I, $p_I = e = 40$, λ = 405 mμ, *recorder divisions*	26	35	30	*
$t_{\frac{1}{2}\ off}$, *sec.*	26	17	27	*
k_3', $sec.^{-1}$	2.0	2.3	1.8	
Complex II, $p_{II} = e = 30$ divisions, λ = 435 mμ, *recorder divisions*	15	5		

* Enzyme-substrate compound does not form and optical density of the Soret band slowly decreases.

datic activity is not decreased at pH 3.65 compared with that at pH 7.0. In the latter experiments, the activity was determined from the kinetics of complex I and was calculated according to Equation 12.

In addition, no appreciable change in the rate of formation (k_1) or breakdown (k_3) of the primary catalase-peroxide complexes was observed in these experiments.

The lack of pH effect in the hydrogen peroxide reaction at pH greater than 3.8 (10) may be explained by Equation 16

$$\mathrm{Cat(OH)_4 + HOOH \underset{k_2}{\overset{k_1}{\rightleftarrows}} Cat(OH)_3OOH + H_2O} \qquad (16)$$

with the same arguments as in the case of catalase cyanide (11). Down to pH 3.8, catalase is mainly in the form of the hydroxide compound, and hydrogen peroxide is mainly in the form of the undissociated molecule ($K = 1.55 \times 10^{-12}$ (12)). On increasing the pH, the free catalase concentration decreases as rapidly as the peroxide ion concentration is increasing, and there is no effect of pH upon the dissociation constant between $3.8 < \mathrm{pH} < 11.8$. Above pH 8.8, however, changes in catalase probably occur (see the paragraph below). The alkyl hydrogen peroxides are also weak acids (pK ~ 11 for methyl hydrogen peroxide[6]) and the combination of catalase and alkyl hydrogen peroxide is written

$$\mathrm{Cat(OH)_4 + 4\,HOOR \underset{k_2}{\overset{k_1}{\rightleftarrows}} Cat(OOR)_4 + 4H_2O} \qquad (17)$$

The experiments show, however, no measurable decrease of activity at pH 3.65, at which the hydroxyl group is dissociated from catalase. A similar effect has been observed in peroxidase when the hydroxyl group is dissociated without loss of activity (13). In acid solutions, the value of k_2 would be expected to increase greatly owing to the reaction of hydrogen ions with the peroxide complex. In the case of catalase, the pH cannot be decreased much below the pK of the hydroxyl group (3.8) without denaturation of catalase protein. Thus only a relatively small increase of k_2 would be obtained and would not affect the properties of catalase as measured in the activity test where $k_3 >> k_2$ and $K_m = (k_2 + k_3)/k_1$. In the case of horseradish peroxidase, the corresponding pK = 5.0, and more complete data have been obtained and are discussed later (14).

In alkaline solutions, there is a decrease of activity at pH 8.8 and a complete loss of activity below pH 11. Below pH 11, the catalase-peroxide complex does not form, and the enzyme is slowly destroyed. Catalase, as contrasted with peroxidase, has no alkaline form; yet the loss of activity of catalase and peroxidase in alkaline solutions is due to the same reason: neither can form a peroxide complex (14). In peroxidase, the change of spectrum indicates a change of a heme linkage. Although catalase shows no measurable spectral shift, a change of heme linkage in alkaline solutions is possible.

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[6] Unpublished data.

The records of Fig. 9 were obtained by the addition of methyl hydrogen peroxide to a catalase solution contained in an open cuvette, as in the case of Fig. 8. These data show that the conversion from complex I to inactive complex II proceeds 3 times as fast at pH 3.5 as at pH 7.0. Thus activity tests which do not take into account the increased formation of complex II in acid solutions give too low an activity. The more rapid inactivation of catalase in the presence of hydrogen peroxide in acid solutions caused by the more rapid formation of catalase hydrogen peroxide II has recently been observed (15).

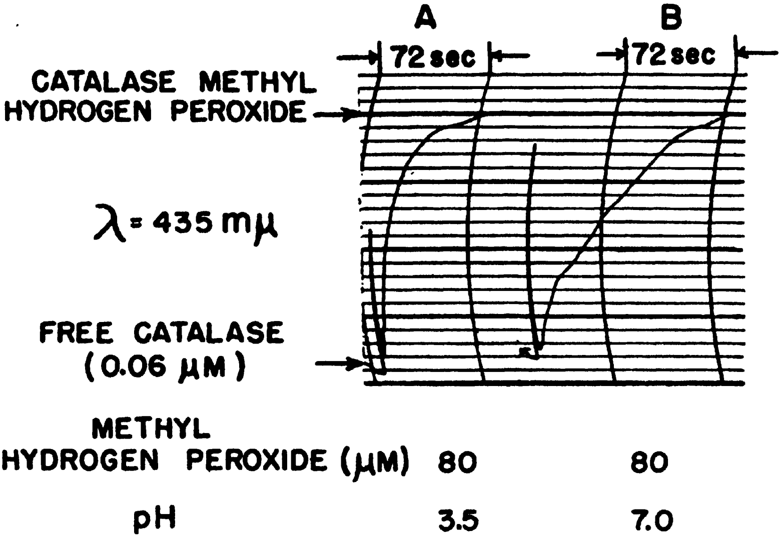

FIG. 9. The increase of the rate of formation of the secondary catalase-methyl hydrogen peroxide complex in acid solutions. Curve A, pH 3.5, 0.001 M phosphate; Curve B, pH 7.0, 0.01 M phosphate buffer. 0.24 μM of hematin iron horse blood catalase, 80 μM of methyl hydrogen peroxide, 435 mμ (Experiment 258). The experimental technique is the same as that used in Fig. 8.

DISCUSSION

Since dilute solutions of the alkyl hydrogen peroxides and their reaction products give very little absorption in the visible or ultraviolet region, and the polarographic technique used elsewhere (16) is apparently insensitive to the alkyl hydrogen peroxides, and the titration of 5 to 50 μM of peroxide is not possible with ordinary methods, the rate of decomposition of the dilute alkyl hydrogen peroxides by catalase has been calculated from the kinetics of compound I in a manner previously proved valid for peroxidase and leucomalachite green (5) and recently shown to be valid over a wide range for the system peroxidase-hydrogen peroxide-ascorbic acid (17). This method of calculation of the activity is valid for an enzyme-substrate compound that obeys the Michaelis theory.

Spontaneous Decomposition of Complexes—Both of the primary catalase alkyl hydrogen peroxides decompose spontaneously, and the kinetics of these reactions are governed by the concentration of the primary compound in accord with the Michaelis theory.

The spontaneous breakdown of the primary complexes is very slow and does not increase appreciably with the alkyl hydrogen peroxide concentration. This is not, therefore, the catalatic reaction by which catalase destroys hydrogen peroxide so rapidly. The velocity constant is about the same as that for the slow breakdown of horseradish and lactoperoxidase peroxides (5, 18) or catalase hydrogen peroxide (4).

Although spontaneous decomposition is a common property of the hematin peroxide compounds (methemoglobin, horseradish peroxidase, lactoperoxidase, etc.), there is not much known about the exact mechanism of this reaction. Keilin and Hartree (19) have clearly shown that no oxygen is evolved in the spontaneous decomposition of strong solutions of methemoglobin and hydrogen peroxide, or ethyl hydrogen peroxide; therefore a peroxidatic reaction is involved. The nature of the acceptor in such a reaction is unknown.

Reactions of Catalase-Peroxide Complexes with Alcohol—The reactions of the primary catalase-alkyl hydrogen peroxide complexes with alcohols now provide the second example of true Michaelis intermediate compounds, for their concentration accurately determines the rate of alcohol oxidation by catalase. The catalase complexes actually provide a simpler example of a Michaelis intermediate than do the peroxidase complexes (5). In catalase kinetics, the reaction of the primary complexes with the acceptor is usually the rate-determining step, while, in peroxidase kinetics, the reaction of the secondary complexes with the acceptor is usually the rate-determining step (17, 18).

With ethyl and methyl alcohol as acceptors, the Michaelis theory completely explains the activity of the primary catalase alkyl hydrogen peroxides over a wider range of enzyme, substrate, and acceptor concentrations than had previously been possible with peroxidase, hydrogen peroxide, and ascorbic acid (5). New techniques (17) have considerably extended the range of the tests with peroxidase.

Since the value of k_4 for the catalase alkyl hydrogen peroxides represents the reaction velocity of each of the three or four independent catalase-hematin peroxide complexes with alcohol, these values are directly comparable with those obtained for the reaction of catalase hydrogen peroxide with alcohols, provided the mechanisms employed in these calculations have accounted for all experimental factors. The total turnover of alcohol with catalase alkyl hydrogen peroxides is, of course, 3 or 4 times that obtained with catalase hydrogen peroxide, depending upon the number of intact hematins in the catalase used.

The values of k_4 for the reactions of catalase hydrogen peroxide and catalase methyl hydrogen peroxide with alcohols are about the same, in accordance with studies of horseradish peroxidase. Thus the alkyl substituent of the peroxide group does not interfere with the reaction with the acceptor. In fact, the values of k_4 obtained for catalase ethyl hydrogen peroxide are greater than those for the hydrogen peroxide complex.

The saturation effects shown clearly in the kinetics of oxidation of alcohols by catalase hydrogen peroxide (4) have not been demonstrated here, and it is not known whether such a saturation effect exists in these reactions.

There is no correlation between the ability of acceptors to replace the heme-linked hydroxyl group of catalase (10) and their reactivity with the catalase-peroxide complex. Catalase has a relatively high affinity for formate compared with methanol; yet these 2 molecules are oxidized at about the same speed by catalase peroxides. Furthermore, formate is the only one of nine anion inhibitors which is oxidized. Catalase acceptors need not be anions and need not replace the heme-linked hydroxyl group of Agner and Theorell (10).

Reaction Products—According to the discussion above, the kinetics of the spontaneous decomposition of the catalase-alkyl hydrogen peroxide complexes according to Equation 4 is not a "catalatic" reaction of the type

$$2C_2H_5OOH \rightarrow 2C_2H_5OH + O_2 \qquad (18)$$

Chemical data support this view, since no oxygen is evolved in this reaction (1, 3). Thus Equation 4 may follow this course

$$C_2H_5OOH \rightarrow CH_3CHO + H_2O \qquad (19)$$

Both Stern (1) and Keilin and Hartree (3) have obtained qualitative tests for aldehyde formation in the reaction of catalase, ethyl hydrogen peroxide, and ethanol according to Equation 3. Ethanol was probably present in Stern's experiments (see Chance (9)). Since no oxygen is evolved, the ethyl hydrogen peroxide is probably reduced to ethanol, and the acetaldehyde is produced by oxidation of ethanol. The catalytic decomposition of diethyl peroxide gives acetaldehyde and probably alcohol (20).

Although it was not known at the time, the oxidation that Keilin and

Hartree (3) obtained with BaO_2 was due to catalase hydrogen peroxide and that with ethyl hydrogen peroxide was due to catalase ethyl hydrogen peroxide I. No evidence has been found to support Keilin's statement that ethyl hydrogen peroxide (or indeed methyl hydrogen peroxide) decomposes into hydrogen peroxide; our solutions are stable for months, and any hydrogen peroxide formed would be immediately detected by the formation of catalase hydrogen peroxide (6).

There are three unusual types of reactions that can occur in the oxidation of alcohols by the catalase-alkyl hydrogen peroxide complexes according to Equation 5.

First, the reduction of the alkyl hydrogen peroxide molecule bound to catalase hematin probably gives an alcohol which replaces the alcohol molecule oxidized to aldehyde. In the following equation,

$$\mathrm{Cat(OOC_2H_5)_4} + \mathrm{4C_2H_5OH} \xrightarrow{k_4} \mathrm{Cat(OH)_4} + \mathrm{4C_2H_5OH} + \mathrm{4CH_3CHO} \qquad (20)$$

the concentration of ethanol is constant at its initial value. Thus the value of k_4, calculated on the basis of the initial ethanol concentration, will be correct even when the ethyl hydrogen peroxide concentration is about equal to the ethanol concentration, as shown in Fig. 4. A small initial amount of ethanol present in the ethyl hydrogen peroxide solution could maintain a constant reaction velocity while all the ethyl hydrogen peroxide is being decomposed (9). Thus this reaction is analogous to the cyclic oxidations of Keilin and Hartree (3).

Second, the alcohol formed from the alkyl hydrogen peroxide can be more reactive than the alcohol used as an acceptor; then the reaction velocity will increase during the reaction. In Fig. 7, the reaction velocity is relatively higher with a small initial concentration of *n*-propanol than with the higher concentrations. This effect is also seen in the values of k_4 given in Table IX.

Third, the oxidation product of the acceptor can be an acceptor. In separate experiments (15), evidence has been obtained for the formation of formaldehyde from methanol and formic acid from formaldehyde, all of which react rapidly with catalase peroxides. In the reaction,

$$\mathrm{Cat(OOCH_3)_4} + \mathrm{4CH_3OH} \xrightarrow{k_4} \mathrm{Cat(OH)_4} + \mathrm{4CH_3OH} + \mathrm{4CH_2(OH)_2} \qquad (21)$$

the total concentration of acceptor increases, and the reaction would be autocatalytic. But no effect is observed in Table II, because the initial methanol concentration is much larger than the methyl hydrogen peroxide concentration. The initial methyl hydrogen peroxide concentration required to produce enough formaldehyde to double the rate found in Table II is so large that much of compound I would be converted to compound II before the reaction was complete.

Accessibility of Catalase Hematin—The reaction velocity of catalase with peroxides decreases 30-fold from hydrogen peroxide to methyl hydrogen peroxide and a further 50-fold from methyl to ethyl hydrogen peroxide, whereas no comparable decreases are observed in the reactions of horseradish and lactoperoxidase with these peroxides (18, 19). Although ethanol, methanol, formaldehyde, and formate react with catalase peroxides at about the same rate, larger molecules react much more slowly; the decrease of reaction velocity with *n*-propanol is 60-fold.[7] These steric effects are due to the catalase protein and not to the hemin, since horseradish and lactoperoxidase hematin reacts rapidly with large substrate and acceptor molecules. Because of the sensitivity of hematin to hydrogen peroxide and the irreversibility of the catalase-splitting experiments, Granick (21) has suggested that the whole hematin group is well hidden by the catalase protein. These data provide some experimental support for Granick's views.

SUMMARY[8]

1. The primary compounds of catalase with methyl or ethyl hydrogen peroxide are formed by the bimolecular combination of all the catalase hematins with the alkyl hydrogen peroxides. The reaction velocity constants increase with decreasing size of the alkyl hydrogen peroxide, 2×10^4 M^{-1} × sec.$^{-1}$ for catalase ethyl hydrogen peroxide I and 0.85×10^6 M^{-1} × sec.$^{-1}$ for catalase methyl hydrogen peroxide I and are considerably less than the velocity of the formation of catalase hydrogen peroxide (3×10^7 M^{-1} × sec.$^{-1}$).

2. The secondary compounds do not form until an appreciable amount of the primary compound has formed. They are not, however, formed in a simple monomolecular conversion from the primary to the secondary compounds; the velocity of the formation of the secondary compounds increases with the alkyl hydrogen peroxide concentration, but not enough to follow a second order equation.

3. Both primary compounds decompose spontaneously into free catalase at about the same rate as horseradish peroxidase hydrogen peroxide or catalase hydrogen peroxide. The velocity constant for catalase methyl hydrogen peroxide I is 0.016 sec.$^{-1}$ and for catalase ethyl hydrogen peroxide I 0.04 sec.$^{-1}$. Both these velocity constants can be measured in such dilute alkyl hydrogen peroxide that alcohol present in the alkyl hydrogen peroxide would make a negligible contribution to the reaction velocity.

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[7] The decrease of reaction velocity is much greater than that due to the decreased number of collisions of the larger molecules with the catalase hematin, since the collision number varies inversely as the square root of the molecular weight.

[8] The velocity constants given in this summary were obtained at temperatures between 25–30°.

4. The Michaelis theory accounts for the kinetics of the spontaneous decomposition of the primary compounds. On the basis of kinetic data, the Michaelis constant for catalase methyl hydrogen peroxide I is 2×10^{-8} M and 2×10^{-6} M for catalase ethyl hydrogen peroxide I. Steady state equilibrium measurements give 1×10^{-7} and 3×10^{-6} M respectively. The affinity of catalase for methyl hydrogen peroxide is about the same as that of horseradish peroxidase for hydrogen peroxide.

5. The secondary compounds decompose very slowly in dilute alkyl hydrogen peroxide solutions; the velocity constants are about 4×10^{-5} sec.$^{-1}$ and 2.3×10^{-4} sec.$^{-1}$ for the methyl hydrogen peroxide and ethyl hydrogen peroxide compounds respectively. On the basis of kinetic data, the equilibrium constants of the corresponding secondary compounds are 1.2×10^{-7} and 2.0×10^{-6} M, values which are very nearly equal to those of the primary compounds. The secondary compounds can, therefore, be inhibitors of the activity of the primary compounds.

6. The primary compounds react peroxidatically towards alcohols. Catalase ethyl hydrogen peroxide I is responsible for the oxidation of ethanol to acetaldehyde qualitatively measured by Keilin and Hartree (3).

7. Catalase ethyl hydrogen peroxide I reacts with methyl, ethyl, and *n*-propyl alcohols at rates which are somewhat larger (2100, 2100, and 33 M^{-1} × sec.$^{-1}$ respectively) than the corresponding reactions of catalase hydrogen peroxide. The relative activities of the two enzyme-substrate compounds towards the three alcohols is the same. Catalase methyl hydrogen peroxide I reacts with ethanol, methanol, and *n*-propanol at approximately the same rates as does the hydrogen peroxide complex. The catalase-peroxide complexes also oxidize formaldehyde (probably as methylene glycol) and formate. The substituent attached to the peroxide group has very little effect upon the activity or the specificity of catalase towards alcohols.

8. The reaction kinetics of both primary compounds obey the extended Michaelis theory for the peroxidatic activities over a wide range of alkyl hydrogen peroxide and ethanol concentrations. These compounds furnish the second and third examples of enzyme-substrate compounds which have been studied directly and which have been proved to follow the Michaelis theory.

9. The reaction velocity constants for the formation of the primary compounds and for their reactions with ethanol are unaffected by a change in the degree of saturation of the catalase-alkyl hydrogen peroxide complexes, and it is concluded that the intact catalase hematins have identical reactivities. The total peroxidatic activity of liver and blood catalases is proportional to the number of intact hematin groups per catalase molecule.

10. The peroxidatic activity of catalase is not decreased on changing the pH from 7.0 to 3.65, and the combination of catalase and alkyl hydrogen peroxides follows the equation,

$$\mathrm{Cat(OH)_4} + \mathrm{4HOOR} \longleftrightarrow \mathrm{Cat(OOR)_4} + \mathrm{4H_2O}$$

in the range $3.8 < \mathrm{pH} < 9$. Below pH 11 catalase loses its ability to form the peroxide complexes and is therefore inactive.

11. At pH 3.5, the velocity of formation of the secondary complexes is several times as rapid as at pH 7.0 and explains the greater inactivation of catalase in acid solutions.

12. The peroxidatic activity of the secondary compounds is negligible in dilute solutions. The total peroxidatic activity of catalase is inhibited by the formation of compound II, but the activity of that portion of catalase in the form of compound I is constant.

13. Both the formation of the catalase peroxides and their reactivities towards alcohols increase with decreasing size of the peroxide and the alcohol molecules. It is concluded that the alcohols attach to or near to the hematin peroxide group and that the iron atom of catalase hematin is not readily accessible to large molecules.

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Note Added in Proof—The recent confirmation of the conversion of carbon monoxide to carbon dioxide in the intact animal (Clark, R. T., Stannard, J. N., and Fenn, W. O., *Science*, **109**, 615 (1949)) leads us to suggest that the production of formate from carbon monoxide and its oxidation to carbon dioxide in the presence of catalase and peroxides provide a possible biochemical explanation for their results.

THE ENZYMATIC EQUILIBRIA OF PHOSPHO(ENOL)PYRUVATE*

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Phospho(enol)pyruvate, which was discovered by Lohmann and Meyerhof in 1934 (1), forms enzymatic equilibria both with phosphoglyceric acid and with pyruvate and ATP.[1]

$$\text{D-3-Phosphoglyceric acid} \rightleftarrows \text{D-2-phosphoglyceric acid} \rightleftarrows \text{phosphopyruvic acid} + H_2O \quad (1)$$

$$\text{Phosphopyruvic acid} + \text{ADP} \rightleftarrows \text{pyruvic acid} + \text{ATP} \quad (2)$$

The reversibility of reaction (2) was discovered by Lardy and Ziegler (2), who showed that the failure of Meyerhof *et al.* (3) to demonstrate the back-reaction with P^{32} was due to the absence of K ion. In the meantime it had been found by Boyer *et al.* (4) that K ion is indispensable for the transphosphorylation of phosphopyruvate with ADP.

We have confirmed the results of Lardy and Ziegler, and have determined the equilibrium constant of reaction (2). Lardy's mixture of triose phosphate, ATP, cozymase, inorganic phosphate, and phosphopyruvate or pyruvate, in the presence of dialyzed acetone powder extract of muscle can be used only for a demonstration of the reversibility of reaction (2), with or without P^{32}. It does not, however, allow one to calculate the equilibrium constant, as has been erroneously assumed by some authors. If phosphopyruvate is synthesized from pyruvate by the ATP which forms continuously in the "coupling reaction" of glycolysis, the amounts formed depend on the time of reaction and the affinities of the various reactants in a complicated way; in particular, the relation of phosphopyruvate to pyruvate cannot be used for a calculation of the equilibrium constant.

A determination of the equilibrium constant is only possible when the isolated reaction (2) is studied. In preliminary experiments we have used pyruvate labeled with C^{14} and measured the speed with which non-radioactive phosphopyruvate takes up C^{14} in the presence of dialyzed acetone

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[1] ATP = adenosine triphosphate; ADP = adenosine diphosphate.

powder extract of muscle. This has been compared with the speed of the back-reaction with the same extract. With this method only the order of magnitude of *K*, but no final values, could be obtained. We therefore purified the specific transphosphorylase responsible for reaction (2) until the interfering enzymes were removed and measured the *K* directly by determining the concentration of all reactants in the state of equilibrium. This necessitates the determination of very small amounts of phosphopyruvate and ADP in the presence of a large excess of pyruvate and ATP. Adequate methods to accomplish this are described below, and the final *K* values obtained are reported. It was also necessary to redetermine the equilibria of reaction (1) for an evaluation of the energy difference of the phosphate bonds in phosphoglycerate, phosphopyruvate, and ATP, and for comparison with the known data of the literature (5, 6). The value for the equilibrium of enolase

$$H_2O + \text{phosphopyruvate} \rightleftarrows \text{2-phosphoglyceric acid}$$

found by Warburg and Christian (7) with pure enolase was appreciably at variance with that described earlier (8–10).

Our present value confirms those found formerly. The energy differences for all the compounds in the reaction sequence (reactions (1) and (2) above) now seem clearly established.

Materials and Methods

ATP was prepared by the method of Kerr (11). After the first precipitation with barium acetate and alcohol, the material was thrice redissolved in HCl and reprecipitated with barium acetate alone (pH 4.5). The ATP was routinely analyzed by measuring the ratio of 7 minute P to total P and also with hexokinase (*cf.* Kielley and Meyerhof (12)), but it was found that the ADP content in ATP was best determined by allowing a large quantity of the ATP to react with an excess of phosphopyruvate in the presence of the transphosphorylating enzyme of reaction (2), prepared according to Kubowitz and Ott (13). The decrease in phosphopyruvate is equal to the ADP originally present. (Since the equilibrium lies far to the side of ATP and pyruvate, the reaction is complete.) The ATP preparations contained 2 to 10 per cent ADP (based on 7 minute P; the mole fraction of ADP is nearly twice as great).

ADP was prepared from ATP by means of purified myosin from rat muscle. When the ATP had been completely split to ADP, the myosin was removed with trichloroacetic acid, and the ADP precipitated at pH 7 with barium acetate and 2 volumes of alcohol. The inorganic P was removed by dissolving the ADP in HCl, precipitating the barium with sodium sulfate, adding a quantity of magnesia mixture equivalent to the inorganic phosphate, and making the solution alkaline with ammonia. After removal of the precipitate, the ADP was recovered as the magnesium salt by adding more magnesia and 4 volumes of methyl alcohol. The magnesium salt was redissolved in dilute acid, barium acetate added, and the barium salt precipitated at pH 5.5 with 2 volumes of ethyl alcohol. In this way it is possible to prepare a barium salt of ADP nearly free of inorganic phosphate. The ADP was analyzed by measuring the ratio of 7 minute P to total P, which was 0.502. ADP was also tested with hexokinase.

The D-3-phosphoglycerate was an acid barium salt prepared from yeast maceration juice and recrystallized several times. It had an $[\alpha]_D$ of the free acid in N HCl equal to −13.2°. This rotation, which is 9 per cent lower than the highest rotation formerly found (−14.5°), is not due to partial racemization, because the sample was 100 per cent fermentable, but is probably due to a small admixture (3 per cent) of D-2-phosphoglyceric acid. We have assumed this in calculating our experiments.

Phosphopyruvate was a synthetic preparation of the barium salt made according to Kiessling (14) about 10 years ago. Free pyruvate and inorganic phosphate were removed by the same procedure as that used for ADP. 9 gm. of the original phosphopyruvate yielded 5.8 gm. of the purified salt, free of inorganic phosphate and pyruvate, and contained 5.0 per cent of organic P, of which 4.8 per cent was split by iodine (96 per cent pure).

The phosphate was determined according to the routine method of our laboratory, based on Lohmann and Jendrassik's modification (15) of the method of Fiske and Subbarow (16).

Phosphopyruvate was determined by a modification of the method of Lohmann and Meyerhof (1). A slight excess of 0.1 N iodine was added to the sample, and enough 2 N NaOH added to remove the iodine color After 2 minutes, the solution was neutralized with HCl; the treatment with NaOH and HCl was repeated twice, and the iodine was finally reduced with bisulfite. The iodoform was centrifuged off. This procedure, involving only a short period of alkalinity, splits phosphopyruvate P quantitatively, without decomposing even a trace of ATP. Phosphopyruvate was also determined by the mercuric chloride method (see (1)), and the results were the same as those obtained by the iodine method.

Sodium pyruvate was prepared by the method described by Lardy (17) and was recrystallized from aqueous alcohol.[3]

Pyruvate was analyzed by Procedure B of Friedemann and Haugen

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[3] We thank Dr. J. M. Buchanan of this department for the sample of pyruvate used.

(18) with an Evelyn colorimeter; the readings were made in tubes containing 10 cc. solution with about 20 γ of pyruvate.

Radioactive pyruvate, labeled with C^{14} in the α and β positions, was prepared by the method of Anker (19).[3]

The activities of the pyruvate and phosphopyruvate were determined as follows: The phosphopyruvate was precipitated as the barium salt at pH 8, with 2 volumes of alcohol. The barium salt was redissolved in acid, non-radioactive pyruvate was added, and the barium salt reprecipitated and dried. Since pyruvate does not precipitate under these conditions, the two precipitations serve to give a product free of contamination by the radioactive pyruvate originally present in the incubation mixture. This point was established by pyruvate analyses at each step; only 0.3 to 0.5 per cent of the final phosphopyruvate samples consisted of the initially active pyruvate, although 1 to 2 per cent of inert free pyruvate was present.

Both pyruvate and phosphopyruvate were converted into iodoform with iodine and alkali. This prevents contamination by carbon from any compounds (especially ATP) which do not form iodoform. The iodoform was burned to CO_2 by the method of Van Slyke and Folch (20), and the CO_2 was collected in barium hydroxide to give barium carbonate, which was spread on plates for the radioactivity measurements.

Acetone powder from muscle extract was prepared as previously described (21). It was rubbed up with water and dialyzed for 5 days to remove coenzymes. This preparation was used for the radioactive exchange experiments and for some of the preliminary equilibrium studies with non-radioactive materials. However, it is unsatisfactory for equilibrium measurements because it contains an adenylic acid transphosphorylase, which catalyzes the reaction

$$\text{Adenylic acid} + \text{phosphopyruvate} = \text{ADP} + \text{pyruvate} \qquad (3)$$

The equilibrium studies, therefore, were carried out with the purified enzyme described by Kubowitz and Ott (13). We started with rabbit muscle and carried the purification through the sixth step. The excess salmine was removed by adsorption with $Al(OH)_3$ (*cf.* Warburg and Christian (22)). The enzyme was preserved in half saturated ammonium sulfate, and the inorganic phosphate was removed by dialysis against $\frac{1}{2}$ saturated ammonium sulfate. The resulting preparation is free of the adenylic acid enzyme, and practically free of enolase (we added fluoride to all our reaction mixtures, however), but it still contains some myokinase. Since the ratio of ATP to ADP at equilibrium is always at least 5:1, and frequently much higher, the presence of myokinase is not serious, since only small amounts of the ADP can be converted to adenylic acid. Our equilibrium constants are all given twice, once on the assumption that the myokinase reaction has not taken place at all, and again, on the assumption that the myokinase reaction has gone to completion. The difference between the two figures averages about 15 per cent.

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[3] We thank Dr. Samuel Gurin of this department, and his collaborators, for the preparation of the radioactive pyruvate and the measurement of the activity of the samples.

Results

Equilibrium with Radioactive Pyruvate—When phosphopyruvate and pyruvate are mixed in a molar ratio of about 2:1, and a relatively small amount of ATP added, along with a large excess of transphosphorylase

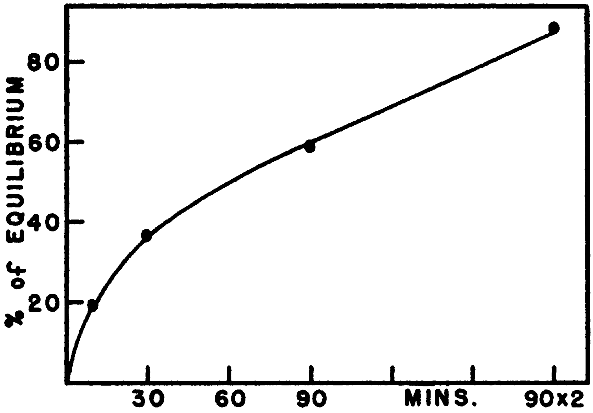

FIG. 1. Uptake of C^{14} by phosphopyruvate from radioactive pyruvate + ATP. Temperature of incubation, 38°. The last point is obtained by incubating for 90 minutes with a double amount of enzyme (8 ml. instead of 4 ml.). The equilibrium is defined as the equal distribution of C^{14} in the pyruvic group of free pyruvate and phosphopyruvate. The enzymatic mixture for each point consists of 11 mg. of radioactive pyruvic acid (neutralized), phosphopyruvate containing 19.4 mg. of pyruvic acid, and 6.81 mg. of organic P, 4 or 8 ml. of dialyzed acetone powder extract of rabbit muscle with 0.13 per cent $NaHCO_3$, 0.03 M KCl, and 0.04 M NaF in a total volume of 22.6 ml.

and the ions necessary for its activity, it may be assumed that reaction (2) will come to equilibrium almost instantaneously. Because the equilibrium lies far to the right side, the attainment of equilibrium involves a reaction of phosphopyruvate with the traces of ADP contained in the ATP. This amount of ADP is, however, too small to diminish the phosphopyruvate in an analytically measurable way. If fluoride is present, along with magnesium and inorganic phosphate, the enolase will also be inhibited (1, 7) and the possibility of transformation of phosphopyruvate into 2-phosphoglycerate will be excluded. Indeed, in all the experiments of this type, the amount of phosphopyruvate remains completely constant throughout the incubation.

If, however, the pyruvate is labeled with C^{14}, a kinetic exchange takes place between pyruvate and phosphopyruvate, and the latter gradually becomes tagged with C^{14}. The curve of this uptake of radioactive carbon is shown in Fig. 1. Complete isotope equilibrium was not obtained even after 90 minutes, with double the usual amount of enzyme (at 38°), and the isotopic exchange was only 60 per cent complete in this time with the normal amount of enzyme. The method is sensitive enough, however, to enable one to calculate the speed of isotope exchange if only a few per cent of the equilibrium amount of exchange has occurred.

At first glance it would appear that one could calculate the equilibrium constant of reaction (2) by determining the rate of isotope exchange and also the rate of reaction between phosphopyruvate and ADP. The isotope exchange would give the rate of phosphorylation of pyruvic acid (see (3)), and the equilibrium constant would be

$$K = \frac{k_{\text{dephosphorylation}}}{k_{\text{phosphorylation}}}$$

This would be true, however, only if the velocities of the forward and back reactions were proportional to the concentrations of the reactants. With most enzyme reactions this is not the case (see Bücher (23) and Meyerhof and Green (24)). It is not the case here, either. ATP inhibits the reaction of phosphopyruvate with ADP, and the reaction between pyruvate and ATP occurs to such a small extent that it is impossible to determine whether or not it is first order with respect to each of the reactants, or whether it is inhibited by any of the substances participating in the equilibrium.

If the results were calculated on the assumption that the reaction velocities are proportional to the concentrations of the reactants, K would be about 500. Because of the uncertainties in the calculation, we refrain from reproducing these experiments.

Equilibrium with Non-Radioactive Pyruvate—Direct equilibrium determinations were made by mixing varying amounts of ATP, ADP, phosphopyruvate, and pyruvate in the presence of the enzyme, with additions of KCl, Mg^{++}, NaF, and sodium bicarbonate buffer. When the system had come to equilibrium, as shown by the fact that the concentrations of the components did not change with time, the phosphopyruvate was determined. Since all the initial quantities were known, and the decrease in phosphopyruvate was equal to that of the ADP, and to the increase of pyruvate and ATP, the final equilibrium concentrations of all participants could be calculated. Table I gives a typical protocol.

The ADP content of the ATP was measured in the same way (see Table II).

The transphosphorylase was routinely tested for activity and for enolase, myokinase, and adenylic acid transphosphorylase by incubating it with phosphopyruvate and other suitable substrates, as summarized in Table III. It will be seen from the final column that enolase and adenylic acid transphosphorylase are absent, but that the myokinase activity is considerable, even when the phosphopyruvate decrease due to the ADP in the ATP is subtracted.

Table I

Direct Equilibrium Determination

10.0 μM of ATP, 3.00 μM of ADP, 2.50 μM of phosphopyruvate, 52.2 μM of pyruvate plus 0.1 ml. of 0.5 M KCl, 0.15 ml. of M NaF, 0.2 ml. of 1 per cent NaHCO$_3$, 150 γ of Mg^{++} incubated at 30° with 0.3 ml. of enzyme (diluted to 1:6). Total volume, 1.85 ml. Reaction stopped with 1 ml. of 15 per cent trichloroacetic acid. 1 ml. samples for direct and iodine P.

Time	Total iodine P	Total direct P	Turnover	Final			K
				ATP	ADP	Pyruvate	
min.	μM	μM	μM	μM	μM	μM	
0	2.50	2.10					
10	0.45	2.24	2.05	12.1	0.95	54.3	
20	0.42	2.26	2.08	12.1	0.92	54.3	1630

Table II

Determination of ADP in ATP

ATP containing 1230 γ of 7 minute P, phosphopyruvate containing 78 γ of iodine P, plus 0.1 ml. of 0.5 M KCl, 0.2 ml. of 1 per cent NaHCO$_3$, 0.15 ml. of M NaF, and 150 γ of Mg^{++} incubated at 30° with 0.3 ml. of enzyme (diluted to 1:6). Total volume, 1.85 ml. Reaction stopped with 1 ml. of 15 per cent trichloroacetic acid. 1 ml. samples for direct and iodine P.

Time	Total iodine P	Total direct P	Decrease in iodine P
min.	γ	γ	γ
0	78	38	
10	48	41	30
20	48	40	30

The ATP therefore contains 30 γ of ADP 7 minute P and 1200 γ of ATP 7 minute P (2.5 per cent ADP).

The results of twelve equilibrium experiments are summarized in Table IV. In Experiments 6, 7, 9, and 11, the equilibrium was approached from the side of ATP plus pyruvate, and phosphopyruvate was formed during the reaction; in the remaining experiments the starting materials contained more than the equilibrium amounts of ADP and phosphopyruvate, and

TABLE III

Test of Transphosphorylase

All samples contain 86 γ of phosphopyruvate P, 0.14 ml. of 0.5 M KCl, 0.16 ml. of 1 per cent $NaHCO_3$, 150 γ of Mg^{++}, and 0.3 ml. of enzyme (undiluted) in 1.4 ml.

Test	Adenylic acid P	ATP pyrophosphate P	ADP pyrophosphate P	M NaF	Time	Final phosphopyruvate P	Turnover P
	γ	γ	γ	*ml.*	*min.*	γ	γ
Activity (enzyme 1:100)			60	0.1	10	52	34
Adenylic acid transphosphorylase	53			0.1	20	86	0
Enolase					20	86	0
Myokinase	27	60		0.1	20	44	42
ADP in ATP		120		0.1	20	78	6

TABLE IV

Direct Equilibrium Determinations

The quantities of all reactants are given in micromoles. Temperature, 30°. The first column of K values represents those actually determined; the second column ($K_{\text{myokinase}}$), the values corrected on the assumption that the myokinase equilibrium has been attained. Total volume = 1.3 – 2.0 ml.

Experiment No.	Initial				Final				K	$K_{\text{myokinase}}$	Myokinase ratio	Time
	Phosphopyruvate	ATP	ADP	Pyruvate	Phosphopyruvate	ATP	ADP	Pyruvate				
												min.
1	2.52	10.1	3.10	50.0	0.39	12.2	0.97	52.1	1680	1920	0.3	10, 20
2	2.52	10.0	3.10	53.8	0.71	11.8	1.29	55.6	800	950	0.2	10, 20
3	2.52	10.0	3.00	54.3	0.44	12.1	0.92	54.3	1630	1850	0.25	10, 20
4	2.65	10.0	2.93	49.8	0.50	12.2	0.78	52.0	1630	1820	0.2	10, 20
5	2.52	19.8	3.00	43.9	0.68	21.6	1.16	45.7	1250	1380	1.0	15, 30
6	0.20	19.7	1.94	81.0	0.39	19.5	2.13	80.8	1900	2280	1.8	20, 40
7	0.00	21.8	2.14	46.0	0.165	21.6	2.31	45.8	2600	3070	4.0	20, 40
8	2.68	18.8	2.71	52.8	0.68	20.8	0.71	54.8	2360	2540	14	15, 30
9	0.00	21.3	2.39	75.1	0.26	21.0	2.65	74.8	2280	2800	30	20, 40
10	2.61	10.3	2.87	50.0	0.52	12.4	0.77	52.1	1610	1800	10	15, 30
11	0.02	21.8	3.96	70.5	0.25	21.6	4.19	70.3	1450	1910	24	20, 30, 40
12	3.03	19.8	6.50	67.0	0.12	22.6	3.65	69.8	2400	3060	5	5, 10, 15
Average (without Experiment 2)									1800 ±425	2115 ±530		

phosphopyruvate disappeared. Essentially the same equilibrium constant was obtained no matter from which direction the equilibrium was approached.

In order to evaluate the experiments exactly, it is important to know the "myokinase ratio," *i.e.*, the ratio of the myokinase actually present in the enzyme to that which would be required to form the equilibrium quantity of adenylic acid (from the ADP) in the incubation period, if the initial rate of formation were maintained throughout the incubation. When this ratio is less than 1, the myokinase equilibrium is obviously not attained. This is the case for Experiments 1 to 5 of Table IV; in the other experiments the myokinase equilibrium probably is attained. However, since the difference between the K values calculated with and without the myokinase correction is within the limits of accuracy of the determination, we have not distinguished between the two sets of experiments. The average of all experiments,[4] from the mean of K_{direct} and $K_{\text{myokinase}}$, is 1960 ± 475.

Enolase Equilibria—In regard to the enolase equilibria of reaction (1), three different equilibria must be considered. If we call the true equilibrium of enolase K_{I}, then

$$K_{\text{I}} = \frac{[\text{Phosphopyruvate}] \times [\text{H}_2\text{O}]}{[\text{D-2-Phosphoglyceric acid}]} \tag{4}$$

(see (7)). The equilibrium of phosphoglyceric mutase would be

$$K_{\text{II}} = \frac{[\text{D-3-Phosphoglyceric acid}]}{[\text{D-2-Phosphoglyceric acid}]} \tag{5}$$

and the complete equilibrium

$$K_{\text{III}} = \frac{[\text{Phosphopyruvate}] \times [\text{H}_2\text{O}]}{[\text{D-3-Phosphoglyceric}] + [\text{D-2-phosphoglyceric}]} \tag{6}$$

K_{I} can be calculated from K_{II} and K_{III}, both of which can easily be determined with dialyzed extracts of muscle.

$$K_{\text{I}} = K_{\text{II}} \times K_{\text{III}} + K_{\text{III}} \tag{7}$$

K_{III} was determined by Lohmann and Meyerhof (1), starting with D-3-phosphoglyceric acid. K_{III} at 20° was 0.39, at 40°, 0.54. K_{III} at 24° is found by interpolation to be 0.42. In order to make sure that this is a thermodynamic equilibrium we repeated the measurement at 24°, in some experiments the pure water being replaced with a mixture of water plus alcohol. Because $c_{\text{H}_2\text{O}}$ was now smaller, the percentage of phosphopyruvate should have been increased. This was actually the case. In the last two columns of Table V, K_{III} is first calculated as the fraction, phosphopyruvate to phosphoglyceric acid, assuming the activity of water = 1, and then calculated with the actual activity of water (K'_{III}).

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[4] The preliminary value ($K \backsim 300$) used in the paper presented to the Federation meeting in March, 1948 (see (25)), was based on experiments with C^{14} and some direct equilibrium measurements with dialyzed muscle extract. These measurements were vitiated by the presence of the adenylic acid transphosphorylase.

The measurements of the enolase equilibrium were carried out with an enzyme made by homogenizing frog muscle with 1½ volumes of water. The extract was allowed to stand for 2 hours at 20° to "inactivate" the coenzyme system and dialyzed for 3 hours before adding it to the substrate, which consisted of 3-phosphoglycerate in a sodium bicarbonate buffer. Potassium and magnesium ions were also present in the reaction mixture.

TABLE V

Rôle of Water in Equilibrium of Enolase

All contain 150 γ of Mg^{++}, 0.1 ml. of 8 per cent $NaHCO_3$, 1 ml. of enzyme, 0.66 ml. of M KCl in a total volume of 2.1 ml. Temperature, 24°. Reaction stopped with 1 ml. of 10 per cent trichloroacetic acid. 1 ml. samples for iodine and direct P.

Experiment No.	Organic solvent	Time	Initial		Inorganic P	Increase inorganic P	Phosphopyruvate P	Phosphoglycerate P	$K_{III} = \frac{\text{phosphopyruvate}}{\text{phosphoglyceric}}$	$K'_{III} = \frac{\text{phosphopyruvate} \times H_2O}{\text{phosphoglyceric}}$
			Phosphoglycerate P	Inorganic phosphate P						
		min.	γ	γ	γ	γ	γ	γ		
1		0	246	7	7		0	246		
		20	246	7	17	10	70	166	0.42	0.42
1a	18%* methanol	7 + 20	246	7	20	13	75	158	0.46	0.43
		0	240	7	7		0	240		
2		20	240	7	14	7	60	167	0.40	0.40
2a	30 %* ethanol	7 + 20	240	7	13	6	71	163	0.44	0.39

* By volume.

After the reaction had come to equilibrium, the enzyme was precipitated with trichloroacetic acid and the mixture analyzed for phosphopyruvate; the phosphoglycerate remaining was calculated as the difference between the initial phosphoglycerate and the phosphopyruvate and inorganic phosphate formed. When equilibria were determined in the presence of organic solvents, the latter were added only after the reaction had been running for 7 minutes; this prevents the solvents from so damaging the enzyme that equilibrium cannot be attained. The results of these experiments are summarized in Table V. It will be observed that the

K'_{III} values in the last column, where the activity of water is taken into account, are actually constant within the experimental error, although the ratio of phosphopyruvate to phosphoglycerate increases when water is replaced with an organic solvent. The activity of water is calculated from its partial vapor pressure.

For measuring K_{II}, various procedures were used formerly, all based on the measurement of optical rotation. Because $[\alpha]_D$ for D-3-phosphoglyceric acid is $-14.5°$, and for D-2-phosphoglyceric acid $+24.3°$ (in N HCl), the percentage of the latter can be calculated in a mixture of both acids, if the total concentration is known as well as the rotation. If $[\alpha']$ is the specific rotation of the mixture with the sign actually found, then in the mixture

$$\text{D-2-Phosphoglyceric acid in } \% = \frac{[\alpha'] + 14.5°}{38.8°} \times 100 \qquad (8)$$

The second possibility consists in measuring the same rotation in the presence of molybdate; $[\alpha]_D$ for D-3-phosphoglyceric acid is $-725°$, and $[\alpha]_D$ for D-2-phosphoglyceric acid $-68°$ (see (10)). The procedure for calculating the percentage of D-2-phosphoglyceric acid is similar to that in the absence of molybdate. The advantages of the second procedure, namely that the specific rotation of D-3-phosphoglyceric acid is 50 times increased by molybdate and that it is about 10 times as high as that of D-2-phosphoglyceric acid, are partly counterbalanced by secondary influences. High salt concentrations diminish the rotation strongly (1 mole of NaCl about 20 per cent), and different samples of molybdate give slight differences. On the other hand the accuracy of the measurement of direct rotation in the absence of molybdate is greater because of the opposite sign for each of the isomers. We therefore preferred to use both procedures, but laid more stress on the results without molybdate.

The concentration of D-3-phosphoglyceric acid can be made so high that the direct reading in a polarimetric 2 dm. tube amounts to $-0.35°$ at the start (2.3 mg. of phosphoglyceric acid P per ml.). By the amount of phosphopyruvic acid formed it can be ascertained that the equilibrium distribution is attained. This is the case in the experiments reproduced in Table VI, if allowance is made for the secondary split of some phosphopyruvate by the concentrated dialyzed muscle extract. The extract cannot be dialyzed longer than 15 hours without loss of its high activity, and traces of ATP and cozymase may be left. The influence of the latter can be checked by iodoacetate, but dephosphorylation by traces of the adenylic system cannot be completely avoided. Some experiments in the presence of fluoride, in which the formation of phosphopyruvate is only partly delayed and not inhibited because of low phosphate concentration (see (7)), give the same result. By equation (8), at 24° K_{II} equals 6. If this value is used for equation (7), K_I becomes 2.9. This is not very different from the K_I = 2.3 found by Akano (9) with a crude enolase preparation containing only traces of phosphoglyceric mutase. However, it is much larger than the value of Warburg and Christian (7) with completely pure enolase, K_I = 1.43. With K_I = 1.43, K_{II} would be 2.6. From equation (8) it follows that in this case $[\alpha']$ would be 0°, independent of the amount of phosphopyruvate formed. But all our present measurements as well as the older ones ((8) vol. 276, p. 248) agree in that in the absence of fluoride the initial rotation decreases to about half the initial value, but not more.

TABLE VI

Equilibrium of Phosphoglyceric Mutase

Experiment No.	Time	Inhibitor added	$\frac{\text{Phosphopyruvic}}{\text{Phosphoglyceric}}$	Per cent inorganic P formed from phosphoglyceric	α°, measured in N HCl	$[\alpha']$	Per cent 2-phosphoglycerate	$[\alpha'']$ in molybdate	Per cent 2-phosphoglycerate with molybdate
	min.					*degrees*		*degrees*	
1	0							665	(3)*
	20		0.377	1.2				612	12
	40		0.377	1.5				600	15
2	0							630	(3)
	30		0.395	3.1				567	14
3	0	Iodoacetate			−0.33	−12.6		562	(3)
	60	Iodoacetate	0.36	4.3	−0.165	−8.7	15	455	22
	100	NaF	0.37	2.0	−0.195	−10.0	12		
4	0	Iodoacetate			−0.32	−12.4			
	30	Iodoacetate	0.405	1.8	−0.16	−8.6	15.2		
	50	Iodoacetate	0.405	2.4	−0.16	−8.6	15.2		
	50	NaF	0.11	0	−0.235	−9.45	13.0		
5	0	Iodoacetate			−0.385	−14.0		608	(3)
	35	Iodoacetate	0.403	1.5	−0.193	−10.1	11.5	562	13
Average...............							13.6		15†

* The initial content of 2-phosphoglycerate in the preparation of 3-phosphoglycerate is assumed to be 3 per cent.

† Average for the molybdate experiments.

This means that there can be not more than 20 per cent 2-phosphoglyceric acid and that K_{II} is at least 4. On the other hand the value of K_{II}, measured by Meyerhof and Schulz (10) exclusively with molybdate, of 9 at 25° is probably too high.

The difference from the values found by Warburg and Christian may be due to secondary influences on the equilibrium by ions or other concomitant substances. With reactions of very small energy, such factors may play a rôle in the position of the equilibrium.

DISCUSSION

The equilibrium constant of 2×10^3 at 30°, equal to 1.65×10^3 at 20°,[5] for the transphosphorylation reaction (2) means that at 20° the difference of the standard free energy of the energy-rich phosphate bonds in phosphopyruvate and ATP amounts to

$$\Delta F^\circ = -RT \log 1650 = -4300 \text{ calories}$$

Although such a large difference had not been expected by us, it agrees excellently with the two independent calculations for the energy-rich phosphate bond in phosphopyruvate: Lipmann (5) −15,850 calories (recalculated for 20°), and Meyerhof (6) for ATP −12,000 calories. (Moreover, the latter value should be corrected because of the potential of DPN:DPNH_2 to −11,500 calories.) This difference of 4300 calories, which was for some time doubtful because of the uncertainties of the calculations, is now experimentally proved. Incidentally, it is about equal to the difference between the energy-rich bonds in 1,3-diphosphoglyceric acid and ATP in which, according to Bücher (23), $K = 3000$ and $\Delta F^\circ = -4600$ calories. The phosphate bonds of enol phosphate and acyl phosphate have therefore practically the same content of free energy. This can well be understood, because both these bonds are formed in intermediates of carbohydrate metabolism and can therefore only be present in minute concentration in the living cell. But nevertheless they must be able to transphosphorylate readily with the adenylic system, which is present in very much higher concentration.

The measurements of the equilibria of enolase and phosphoglyceric mutase, which confirm our older values, prove that at 20° the energy contents of phosphoglyceric acid (reactions (2) + (3)) and phosphopyruvic acid are practically identical. With the K_{II} of isomerization of phosphoglyceric acid = 6, the energy difference ΔF° becomes −1050 calories between the two isomeric acids. This is in accordance with the general rule that the secondary phosphate ester bond has a considerably higher energy content than the primary bond. As Lipmann has shown (5) the bond energy of the enol phosphate is equal to the difference of the free energy between pyruvate plus water and glyceric acid minus the energy of the ordinary phosphate bond in 3-phosphoglyceric acid. The free energy of the latter bond, which we have determined by equilibrium measurements with D-glyceric acid,[6] was in our experiments roughly −3000 calories.

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[5] With the van't Hoff isochore and with the molar heat of reaction −3500 calories (26).

We thank Mrs. Jean R. Wilson for assistance in some of the foregoing experiments.

SUMMARY

The equilibrium of transphosphorylase between ATP and pyruvate is determined partly with the use of pyruvate containing C^{14} and partly with non-radioactive pyruvate. While the C^{14} experiments gave only qualitative results, a series of measurements with the use of purified transphosphorylase and non-radioactive reactants gave an average value of the equilibrium constant of 1950, which corresponds to a ΔF° of −4400 calories (30°).

A redetermination of the equilibria of enolase and phosphoglyceric mutase confirmed the former values. That the enolase equilibrium is a true thermodynamic equilibrium was proved by reducing the content of water with an alcohol-water mixture. The phosphopyruvate concentration in the equilibrium rises accordingly.

For the equilibrium of phosphoglyceric mutase, (D-3-phosphoglyceric acid)/(D-2-phosphoglyceric acid), $K = 6$ was obtained, corresponding to a ΔF° of −1050 calories.

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[6] We thank Dr. E. Baer for an ample amount of D-glyceric acid.

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THE SYNTHESIS OF GLUTAMINE IN PIGEON LIVER DISPERSIONS*

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The enzymatic synthesis of glutamine was studied in the hope that the information obtained might aid in understanding the formation of peptide bonds in biological systems. The structural and energetic similarity of simple amide and true peptide linkages suggested that the mechanisms for their synthesis might also be similar. In addition, glutamine itself is a substance of wide natural distribution and is probably of great importance in the nitrogen metabolism of plants and animals (2).

Krebs (3) found that glutamine was formed from glutamate and ammonia by slices of kidney, brain, and retina of various vertebrate species. Synthesis of the amide was generally dependent on the respiration of the tissue, which presumably served to supply the necessary energy. However, in retina, which possesses a very active glycolytic system, glutamine was produced anaerobically provided glucose was present. Later Örström, Örström, Krebs, and Eggleston (4) reported the formation of glutamine from pyruvate and ammonia by pigeon liver slices.

The present paper describes experiments on glutamine synthesis in pigeon liver dispersions, and the following paper (5) gives the results obtained with tissue extracts, which lead to a clearer picture of the mechanism of formation of this amide.

Methods

Materials—Commercial preparations of sodium L-glutamate, sodium succinate, fumaric acid, L-malic acid, adenine sulfate, adenosine, and adenosine-3-phosphoric acid were employed, the first four being recrystallized before use. Sodium pyruvate was prepared according to Lipmann (6), oxalacetic acid according to Krampitz and Werkman (7), α-ketoglutaric acid according to Neuberg and Ringer (8), *cis*-aconitic anhydride according to Malachowski *et al.* (9), and *dl*-isocitric acid according to Pucher and Vickery (10). L-Glutamine was obtained from beets by the method of Vickery *et al.* (11). Diphosphopyridine nucleotide was isolated from yeast by the procedure of Williamson and Green (12), further purified by phosphotungstic acid precipitation, and assayed manometrically by hydrosulfite reduction (13). Cytochrome *c* was prepared from horse heart according to Keilin and Hartree (14), dialyzed against water, and preserved by lyophilization; it was assayed spectrophotometrically. Adenosine triphosphate was isolated from the muscle of magnesium-anesthetized rabbits as described by LePage (15). Part was converted to adenosine-5-phosphoric acid by the method of Kerr (16). Acidic substances were neutralized with NaOH when solutions were prepared.

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Preparation of Dispersions—Livers from freshly killed pigeons were suspended in ice-cold isotonic medium, dispersed for 1 minute in a small Waring blendor, and filtered through gauze. Generally 12 parts of a solution containing 0.044 M phosphate buffer of pH 7.4 (prepared from disodium phosphate) and 0.067 M KCl were used. 1.0 ml. of such a dispersion in a reaction mixture of 2.0 ml. final volume provided 77 mg. of fresh liver, phosphate in a final concentration of 0.02 M, and KCl in a final concentration of 0.03 M. When necessary for the purpose of an experiment, the composition of the medium and the proportions of medium to liver were varied.

Preparation of Samples—The reaction mixtures were measured into Warburg vessels, with glutamate and ammonia solutions in the side arm and alkali papers in the center well, and oxygen uptake was measured after temperature equilibration and tipping. At the end of the reaction period, an equal volume of 10 per cent trichloroacetic acid was added to the samples in the vessels, and analyses were performed on the filtrates. Initial samples identical in all essential respects with the incubated samples were prepared and deproteinized at the time when the substrates were tipped in. The analytical values given are the differences between the initial and final figures.

For analysis of the samples, an aliquot of the trichloroacetic acid filtrate was transferred to the distilling apparatus described below and neutralized to brom cresol purple with NaOH, and the ammonia was distilled off. The residue in the distilling tube was transferred to a 10 ml. volumetric flask, neutralized with H_2SO_4, and diluted to the mark. Aliquots were analyzed for glutamine by acid hydrolysis or for α-amino acid nitrogen with ninhydrin.

Colorimetric Determination of Ammonia—A blank, standards containing 0.5 to 1.5 μM of ammonia as ammonium sulfate, and unknown samples in the same range were made to a volume of 10.0 ml. 0.5 ml. of Nessler's reagent, prepared according to Vanselow (17), was added, and the colors were read in an Evelyn photoelectric colorimeter, in 18 × 150 mm. tubes, with Filter 420. A plot of *L* values (2 − log of per cent transmission) against the ammonia content of the standards gave a straight line passing through the origin. In replicate estimations the precision was ±1 to 2 per cent. The presence of more than 0.2 m.eq. of acid in the samples resulted in a diminution of the color intensity.

Distillation of Ammonia—Ammonia was separated from samples by a vacuum distillation procedure based on the method of Archibald (18). The modified apparatus used is shown in Fig. 1. The distilling tube *A*, alkali cup *B*, and capillary *C* are identical with those of Archibald's apparatus. The ammonia passes out of the distilling tube through a small trap *D*, which is connected by means of pressure tubing, with glass parts touching, to a receiver head *E*. A 10 ml. volumetric flask *F* is fastened in the outer tube of the receiver head with a rubber stopper. The inner tube, which is connected to the trap, passes through the neck of the volumetric flask and dips into the receiving acid. The side arm on the outer tube of the receiver head is attached to a water aspirator.

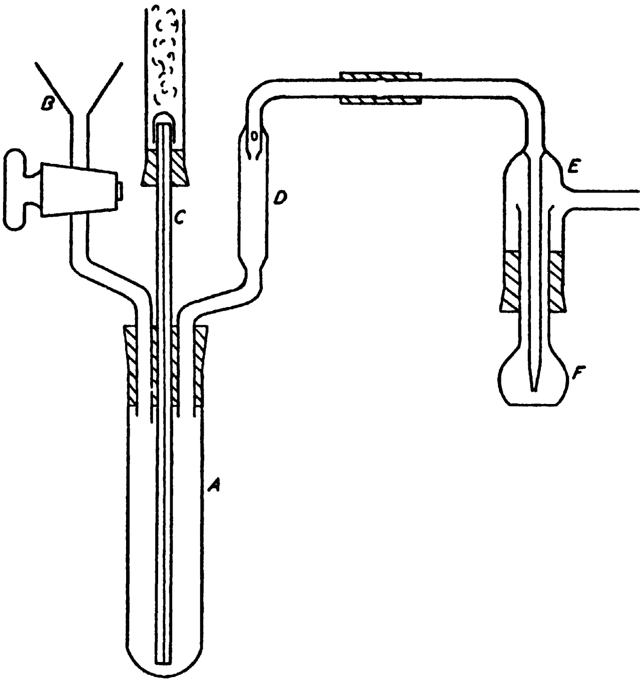

FIG. 1. Ammonia distillation apparatus. *A*, distilling tube made from a 50 ml. centrifuge tube. *B*, cup and stop-cock for admitting alkali. *C*, capillary of marine barometer tubing with trap of 5 N H_2SO_4 on glass wool to wash incoming air; *D*, distillation trap; *E*, receiver head, with side arm for attachment to water aspirator; *F*, 10 ml. volumetric flask.

The sample, nearly neutral and in a volume not greater than 15 ml., is placed in the distilling tube along with a drop of dibutyl phthalate to prevent foaming. 2 ml. of 0.05 N H_2SO_4 are measured into the volumetric flask, and the parts of the apparatus are connected. After application of the vacuum, 2 ml. of borate buffer of pH 10.5 (19) are admitted from the alkali cup, and the distilling tube is lowered into a water bath at 50°. When distillation has proceeded for 8 minutes, the tip of the inner tube of the receiver head is lifted from the acid, and the vacuum is released. The connections are separated, and the tip and bore of the receiver head are rinsed into the volumetric flask. After dilution to the mark, the whole distillate or an aliquot is analyzed for ammonia. The apparatus is steamed out by running two blank distillations before a series of determinations is begun.

0.1 μM to at least 50 μM of ammonia can be quantitatively distilled in this manner. The precision of distilled standards is equal to that of undistilled standards. When correction is made for a small distillation blank, the standard curve for distilled samples is the same as that for undistilled standards. Glutamine present during the distillation yields only about 0.5 per cent of its amide nitrogen as ammonia.

Hydrolysis of Glutamine—The samples containing glutamine were made 1 N in H_2SO_4 by adding 0.25 volume of 5 N acid and were heated 11 minutes in a boiling water bath. After cooling, a volume of 5 N NaOH equal to 0.05 ml. less than the volume of H_2SO_4 used was added, and the samples were transferred to the distilling tube. The ammonia was distilled in the usual manner, the borate buffer used in the distillation being adequate to neutralize the excess acid. The amide nitrogen of glutamine was quantitatively released as ammonia by this procedure. By ninhydrin determinations it was found that after acid hydrolysis glutamine yielded only about 0.2 mole of α-amino acid nitrogen. Therefore, most of the glutamine is converted to pyrrolidonecarboxylic acid by the acid hydrolysis, just as by heating in neutral solution (20).

Determination of α-Amino Acid Nitrogen with Ninhydrin—α-Amino acid nitrogen was determined as ammonia by a method essentially identical with that described by Sobel *et al.* (21). 1 ml. of sample, 0.2 ml. of 1 M citrate of pH 2.5, and 50 mg. of ninhydrin were mixed in an 18 × 150 mm. test-tube and heated 10 minutes in a boiling water bath. Without cooling the tubes, 3 drops of 30 per cent hydrogen peroxide were added, and the heating was continued for 3 minutes. The sample was then cooled, transferred to the distilling tube, and distilled in the usual manner except that 3 ml. of borate buffer were added. The use of stronger alkali and longer distillation times did not alter the amount of ammonia formed. Alanine and glutamic acid yielded 0.96 to 0.97 mole of ammonia by this method. Glutamine also formed 1 mole of ammonia. This result would be expected from the observations of Hamilton (22), who found that at pH 2.5 glutamine undergoes oxidative decarboxylation with ninhydrin much more rapidly than conversion to ammonium pyrrolidonecarboxylate. The ammonia released from glutamine by ninhydrin is therefore derived almost entirely from the α-amino group and only in small part from the amide group.

Results

Standard Reaction System—Experiments in which the conditions of reaction were varied, described in more detail below, led to the selection of the following system as most suitable for studying glutamine synthesis in fresh pigeon liver dispersions: 0.05 M phosphate of pH 7.4, 0.03 M KCl, 0.006 M $MgSO_4$, 0.0001 M diphosphopyridine nucleotide, 6×10^{-6} M cytochrome *c*, 0.01 M sodium citrate, 0.02 M NH_4Cl, 0.05 M sodium L-glutamate, gas phase oxygen, temperature 38°. In such a system, with 77 mg. of fresh weight of pigeon liver in 2.0 ml. final volume and a reaction period of 60 minutes, the following reaction rates were observed (arithmetic mean and range of sixteen experiments): oxygen used, 17.6 μM (13.0 to 22.6); ammonia used, 29.7 μM (19.2 to 43.6); amide formed, 16.2 μM (9.2 to 26.4). If the dry weight of liver is assumed to be one-fifth of the fresh weight, the observed rate of glutamine synthesis corresponds to a value for Q_{amide} of 24. The excess of ammonia used over amide formed is found to be present as α-amino acid nitrogen, as is shown later. The initial ammonia content of the dispersions is zero, and the initial amide content is about 1.4 μM for 77 mg. of liver.

Under the conditions of the standard reaction system, oxygen uptake, ammonia utilization, and amide formation proceed at constant rates for periods up to 90 minutes (Fig. 2) and are nearly proportional to the weight of liver present (Fig. 3).

The rate of hydrolysis of glutamine by the liver dispersions was studied in a test system containing 0.05 M phosphate of pH 7.4, 0.03 M KCl, 0.006 M $MgSO_4$, 0.01 M L-glutamine, and 100 mg. of fresh weight of liver, in a volume of 2.0 ml.; the gas phase was nitrogen and the temperature 38°. Under these conditions glutamine is split at a rate of about 3 μM per 100 mg. of liver per hour. The hydrolysis is almost completely stopped by 0.05 M L-glutamate, an effect previously described by Krebs (3) and Waelsch and Owades (23), and is strongly inhibited by 0.02 M NH_4Cl. Therefore it seems probable that glutamine hydrolysis occurs only at a negligible rate in the standard system for glutamine synthesis.

The various components of the reaction system are considered in more detail in the following sections of the paper.

Glutamate—Little glutamine is formed if glutamate is omitted from the reaction mixture. When the level of added glutamate is varied, a maximum rate of amide synthesis is reached at a concentration of 0.05 M (Fig. 4). The change in ammonia utilization approximately parallels the change in amide formation at different glutamate concentrations. Other substances, particularly members of the tricarboxylic acid cycle, were tested for their ability to replace glutamate in this system. Their relative effectiveness in forming glutamine corresponds to the expected ease of conversion to glutamate by way of α-ketoglutarate (Table I). Glutamate is clearly superior to any other substance rested and is undoubtedly the direct precursor of glutamine. The observation of Örström *et al.* (4) that in pigeon liver slices glutamine is formed more rapidly from pyruvate than from glutamate can probably be explained by the relative impermeability

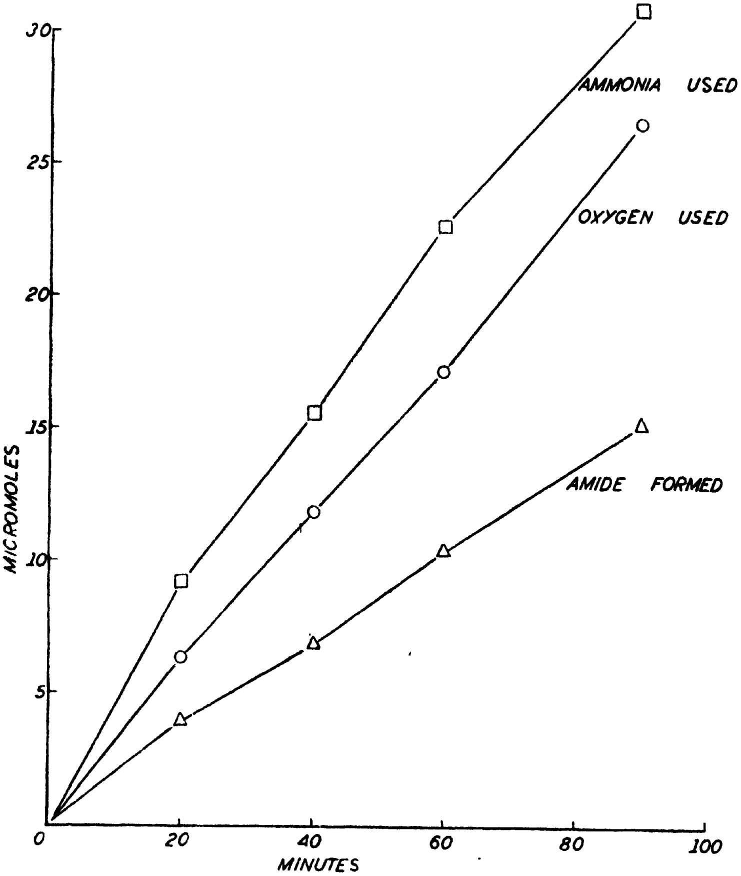

FIG. 2. Time curve of glutamine synthesis. The conditions were those of the standard test system, with 77 mg. of liver in 2.0 ml. final volume.

of the slices to the highly polar glutamate molecule. In the case of most of the nitrogen-free compounds, extra ammonia is used and α-amino acid nitrogen is formed; the sum of amide and α-amino acid nitrogen formed is nearly equivalent to the ammonia which disappears. It is interesting that citrate causes more rapid production of amino acids than the other substances tested. The amino acids have not been identified but presumably consist of alanine and aspartic and glutamic acids, formed by reductive amination and transamination.

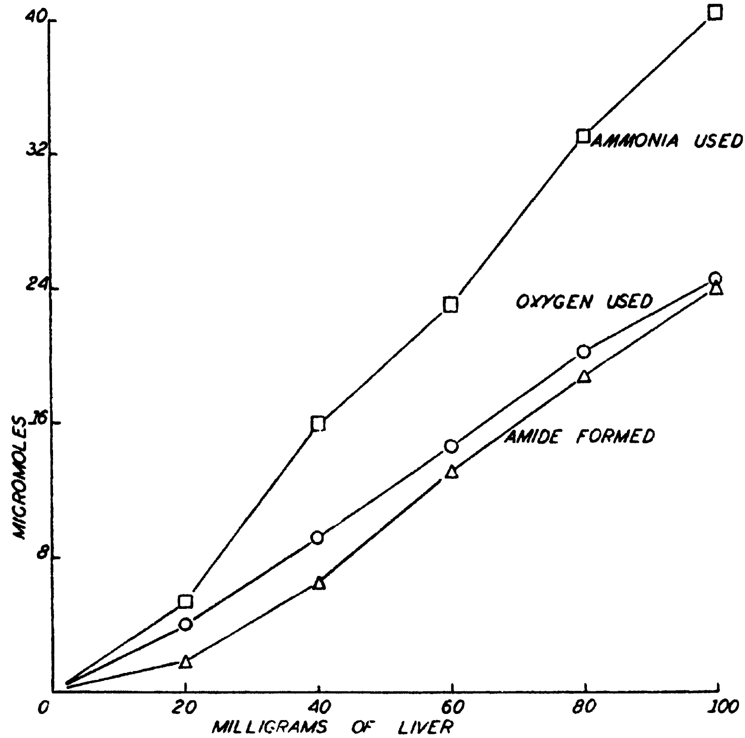

Fig. 3. Glutamine synthesis by varying quantities of liver. The conditions were those of the standard system with the indicated fresh weights of liver in a final volume of 2.0 ml., and a reaction period of 60 minutes.

Ammonia—Variation of the ammonium chloride concentration from 0.01 to 0.03 M does not affect the rate of glutamine synthesis provided not all the ammonia is used. Small amounts of ammonia disappear completely. At intermediate levels the total ammonia uptake may decrease, while amide formation remains unchanged. This indicates preferential utilization of ammonia for formation of amide with glutamate rather than for formation of α-amino acid nitrogen with citrate, probably because of a

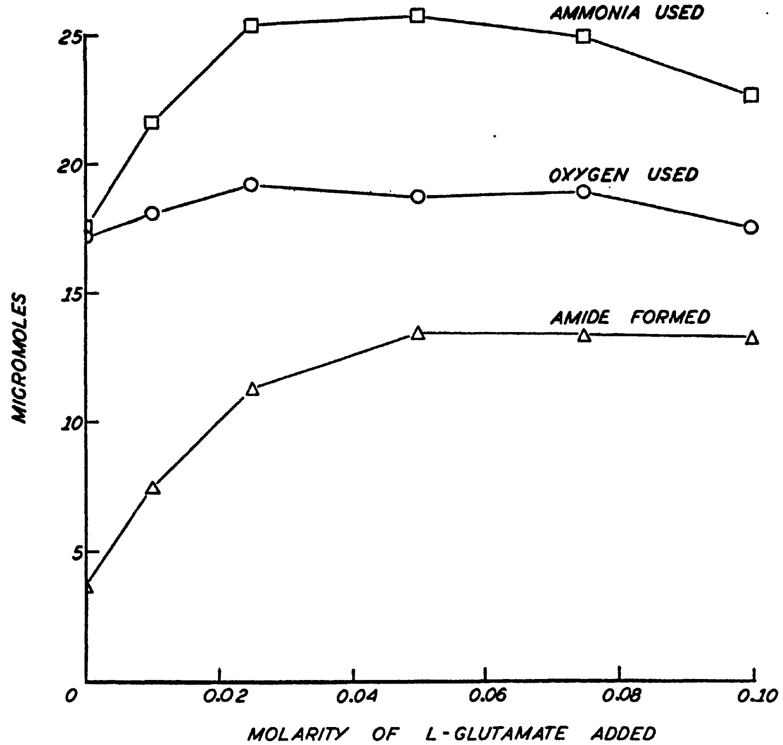

Fig. 4. Glutamine synthesis as a function of glutamate concentration. The conditions were those of the standard system except for variation of the glutamate level. 77 mg. of liver in a final volume of 2.0 ml.; reaction period 60 minutes.

Table I

Carbon Precursors in Glutamine Synthesis

The conditions were those of the standard test system except that glutamate and other carbon precursors were added at a concentration of 0.02 M and citrate was omitted. 77 mg. of liver in 2.0 ml. final volume; reaction period 60 minutes. The data are given in micromoles.

Substrate	Oxygen used	Ammonia used	Amide formed	α-Amino N formed
None	11.5	3.8	−0.2	0.7
Glucose	14.0	1.4	0.1	1.0
Pyruvate	13.7	7.8	1.0	7.2
Succinate	25.3	5.1	0.2	4.4
Fumarate	17.5	6.3	0.6	5.5
L-Malate	17.9	5.0	0.6	6.0
Oxalacetate	15.6	14.0	2.2	13.1
α-Ketoglutarate	16.0	14.0	3.9	10.9
Citrate	15.5	18.0	2.3	16.3
L-Glutamate	16.9	6.9	7.5	−1.3

lower dissociation constant for ammonia and the glutamine enzyme system. Hydroxylamine cannot be substituted for ammonia in the reaction system, since it strongly inhibits respiration.

Phosphate—Omission of inorganic phosphate from the system almost completely abolishes amide formation. A maximum rate of synthesis is observed at a phosphate concentration of 0.05 M (Fig. 5). Oxygen uptake and ammonia utilization show similar variation with phosphate concentration.

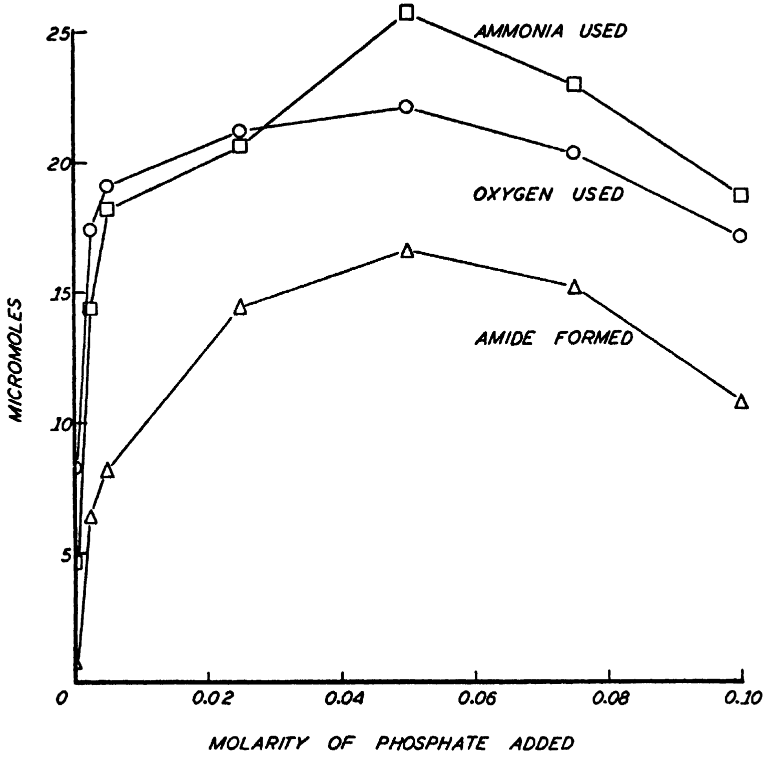

FIG. 5. Glutamine synthesis as a function of phosphate concentration. The conditions were those of the standard system except that the phosphate concentrations were varied; when reduced below 0.05 M, phosphate was replaced by an equimolar quantity of tris(hydroxymethyl)aminomethane buffer of pH 7.4. 77 mg. of liver in 2.0 ml. final volume; reaction period 60 minutes.

pH—Synthesis of glutamine occurs most rapidly when the pH of the reaction mixture is 7.0 (Fig. 6). This is the final pH of the standard system described above with liver dispersion present. Oxygen uptake shows a higher and ammonia utilization a lower pH optimum.

Magnesium Ions—When magnesium ions are omitted from the system, very little glutamine is formed. A magnesium sulfate concentration of

0.006 M is required to give a maximum rate of amide synthesis (Fig. 7). Magnesium ions are also required for maximum rates of oxygen and ammonia utilization.

Oxygen—The rate of glutamine synthesis is decreased when air is substituted for oxygen in the gas phase, and under anaerobic conditions no amide is formed (Table II). The decrease in ammonia utilization on changing the gas phase from oxygen to air is nearly equivalent to the decrease in amide formation. This indicates that the formation of amino acids is not so strictly dependent on high oxygen tension as is amide synthesis.

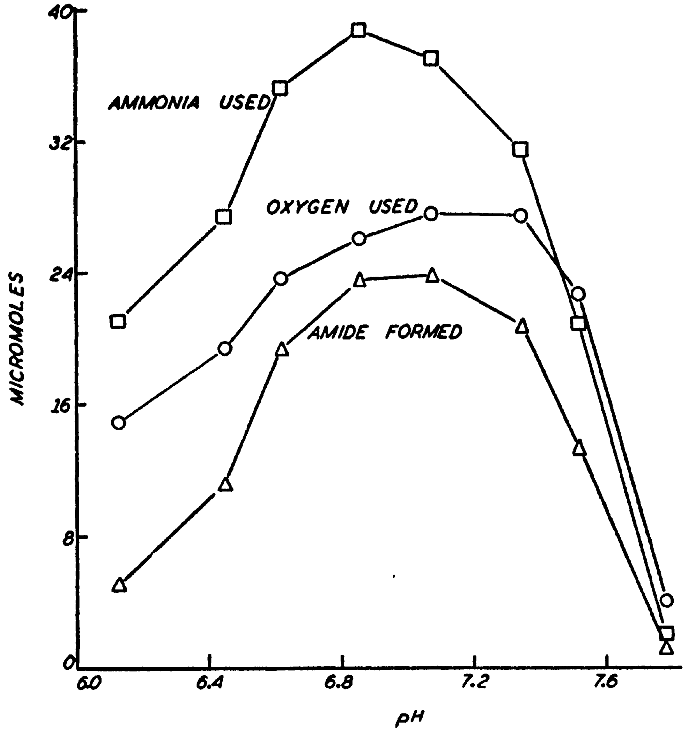

FIG. 6. Glutamine synthesis as a function of pH. The conditions were those of the standard system except that the pH was varied by the addition of small amounts of HCl or NaOH. pH measured in complete reaction mixture including liver dispersions; 100 mg. of liver in a final volume of 2.0 ml.; reaction period of 60 minutes.

Oxidation Substrates—While glutamate is the best substrate for glutamine synthesis in pigeon liver dispersions, addition of other carbon compounds along with optimal amounts of glutamate accelerates formation of the amide (Table III). Succinate, L-malate, oxalacetate, α-ketoglutarate, and citrate increase significantly both ammonia utilization and amide synthesis. Much of the extra ammonia used is converted to α-amino acid nitrogen.

The fact that an equal effect cannot be produced by adding extra glutamate shows that these substances do not act simply by undergoing conversion to glutamate. Presumably their oxidation helps to supply the energy necessary for glutamine synthesis. Citrate is generally the most effective of the oxidation substrates; *cis*-aconitate and *dl*-isocitrate behave as does citrate.

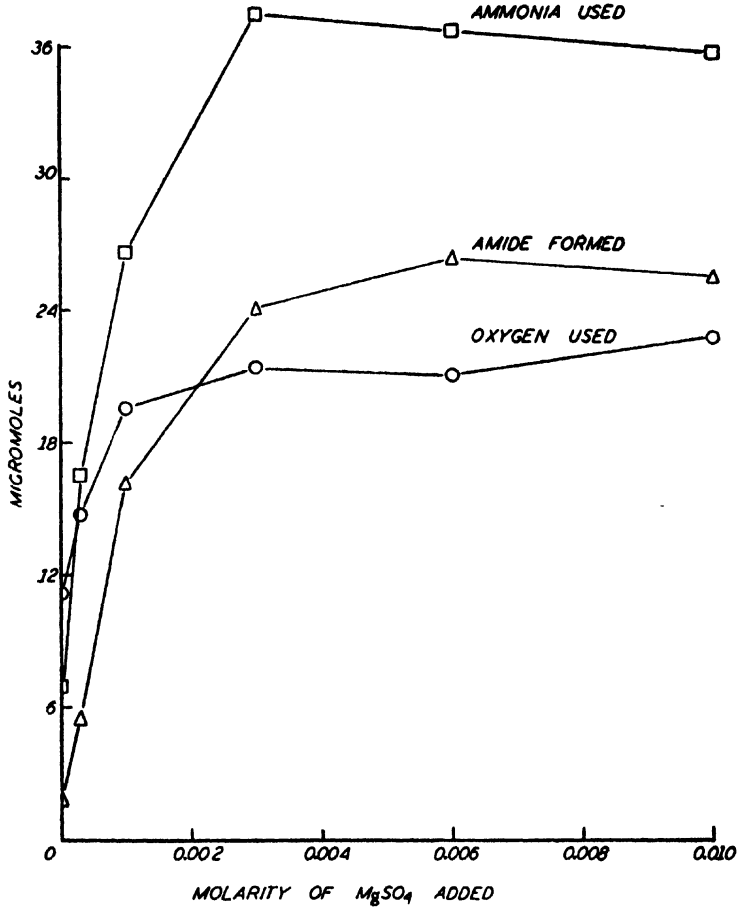

FIG. 7. Glutamine synthesis as a function of $MgSO_4$ concentration. The conditions were those of the standard system except that the $MgSO_4$ level was varied. 77 mg. of liver in 2.0 ml. final volume; reaction period of 60 minutes.

Other Components—The presence of KCl in the test system has only a slight favorable effect on glutamine synthesis. When it is omitted, amide formation decreases about 10 per cent. The optimal concentration of KCl appears to be 0.03 M. Similarly, omission of diphosphopyridine nucleotide lowers the rate of glutamine formation about 10 per cent a andlso depresses oxygen uptake. A concentration of diphosphopyridine nucleotide of 0.0001 M produces a maximum effect on amide synthesis, but the oxygen uptake continues to increase at higher levels. The effect of cytochrome *c* is of the same magnitude as that of KCl and diphosphopyridine nucleotide. 6×10^{-6} M cytochrome *c* is sufficient to give a maximum rate of glutamine formation, but higher concentrations are required for a maximum rate of oxygen uptake. The addition of nicotinamide, diphosphothiamine, or a boiled extract of pigeon liver does not affect amide synthesis.

TABLE II

Requirement of Oxygen for Glutamine Synthesis

The conditions were those of the standard system except that the gas phase was varied. 100 mg. of liver in 2.0 ml. final volume; reaction period 60 minutes. The data are given in micromoles.

Gas phase	Oxygen used	Ammonia used	Amide formed
Oxygen	20.2	32.6	13.1
Air	13.4	25.5	7.6
Nitrogen		4.5	0.0

TABLE III

Oxidation Substrates in Glutamine Synthesis

The conditions were those of the standard system except that various compounds were substituted for citrate, all at a concentration of 0.01 M. 77 mg. of liver in a final volume of 2.0 ml.; reaction period 60 minutes. The data are given in micromoles.

Extra substrate	Oxygen used	Ammonia used	Amide formed
None	21.2	18.8	11.8
L-Glutamate	21.0	18.7	13.1
Glucose	18.1	16.9	11.2
Pyruvate	20.7	24.4	12.5
Succinate	27.2	37.4	16.8
Fumarate	21.4	31.6	13.4
L-Malate	22.9	36.1	14.9
Oxalacetate	23.3	32.0	15.2
α-Ketoglutarate	21.6	34.0	15.2
Citrate	22.6	43.6	18.2

Effect of Adenine Nucleotides on Glutamine Synthesis—The effects produced by addition of adenosine triphosphate or adenosine-5-phosphate to the reaction system depend on the concentration employed and the duration of the experiment. With high concentrations of these nucleotides (0.001 to 0.003 M), glutamine synthesis no longer proceeds linearly with time; instead the rate falls off rapidly after 20 minutes. Therefore, if measurements are made at 20 minutes, it is generally found that adenosine triphosphate or adenosine-5-phosphate in concentrations of 0.0003 to 0.003 M causes acceleration in oxygen and ammonia utilization and in amide for-

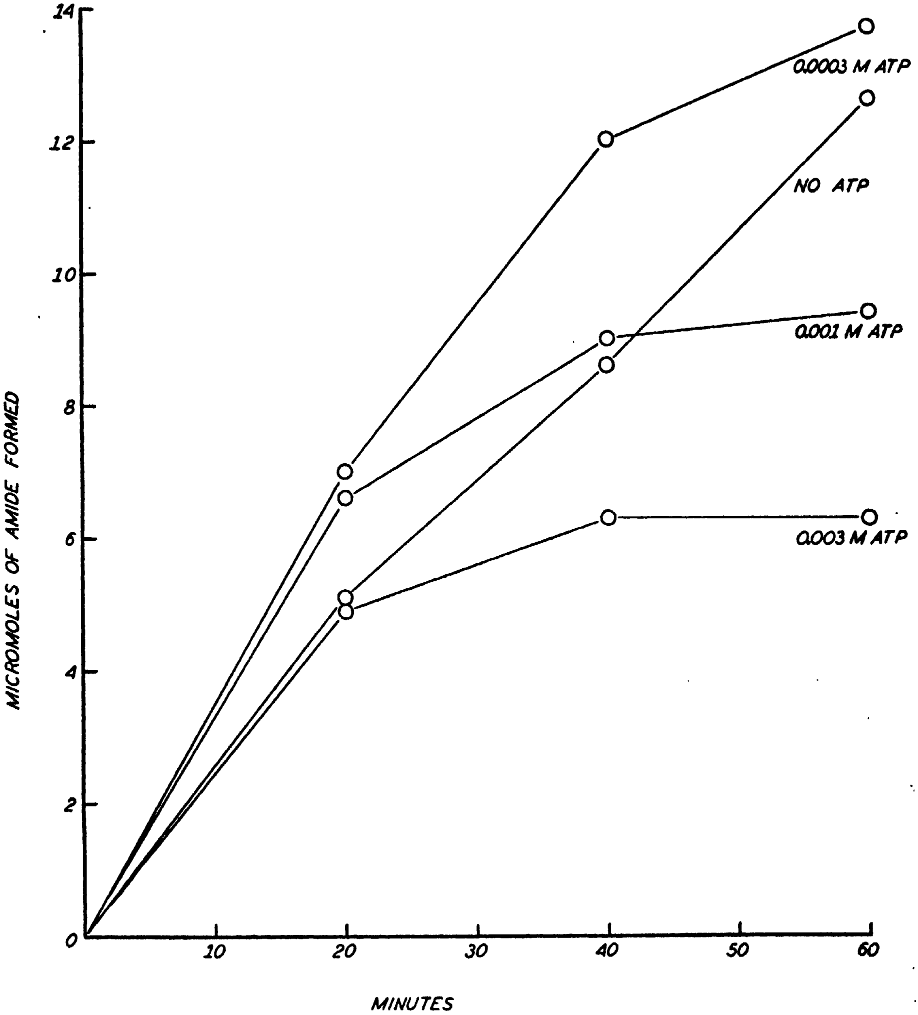

FIG. 8. Effect of adenosine triphosphate on the time curve of glutamine synthesis. The conditions were those of the standard system except for the addition of adenosine triphosphate. 77 mg. of liver in 2.0 ml. final volume; reaction periods as indicated.

mation. After 60 minutes the effect of the lower concentrations of nucleotides is still a stimulation, but with higher levels the rates have fallen off so markedly that the net effect is inhibition of amide formation. This is shown in Fig. 8. These results neither exclude nor prove participation of adenosine triphosphate and adenosine-5-phosphate in glutamine synthesis.

In this connection the rate of hydrolysis of adenosine triphosphate by pigeon liver dispersions was measured. The reaction system contained 0.05 M tris(hydroxymethyl)aminomethane buffer of pH 7.4, 0.03 M KCl, 0.006 M $MgSO_4$, 0.002 M adenosine triphosphate, and 10 mg. of liver per ml.; the gas phase was nitrogen and the temperature 38°. 2 μM of inorganic phosphate were split off in 20 minutes by 10 mg. of liver; this corresponds to about 50 μM per 77 mg. of liver per hour. Therefore the quantities of adenosine triphosphate used in the experiments above could rapidly be destroyed by hydrolysis; any effect produced by the intact molecule of adenosine triphosphate would persist only if the molecule were continuously regenerated.

TABLE IV

Effect of Adenine Derivatives on Glutamine Synthesis

The conditions were those of the standard system except for the addition of the adenine derivatives. 77 mg. of liver in a final volume of 2.0 ml.; reaction period 60 minutes. The data are given in micromoles.

Adenine derivative	Concentration	Oxygen used	Ammonia used	Amide formed
	M			
None		20.2	36.0	22.3
Adenosine triphosphate	0.0003	25.2	39.4	25.2
	0.001	26.1	35.6	24.3
	0.003	24.5	27.7	12.9
Adenosine-5-phosphate	0.0003	23.0	39.7	24.2
	0.001	27.2	40.0	26.2
	0.003	25.9	30.0	16.6
Adenosine-3-phosphate	0.001	19.6	35.0	19.7
	0.003	18.1	29.7	17.1
Adenosine	0.001	22.6	32.5	19.8
	0.003	21.2	25.2	14.2
Adenine	0.001	20.2	30.3	15.6
	0.003	19.4	23.4	10.0

Adenosine-3-phosphate, adenosine, and adenine uniformly cause inhibition of amide formation. Illustrative data are given in Table IV. With these substances the inhibition is apparent from the beginning of the experimental period and the reaction proceeds linearly with time. It is unlikely that the delayed inhibitory effects of adenosine triphosphate or adenosine-5-phosphate are due to hydrolysis to adenosine or adenine, because in some experiments 0.003 M adenosine triphosphate has caused complete inhibition for the period between 40 and 60 minutes (see Fig. 8), while 0.003 M adenosine or adenine has never produced so large an effect.

Inhibitors of Glutamine Synthesis—Sodium fluoride in low concentrations strongly depresses glutamine synthesis in pigeon liver dispersions (Table V). The effect on amide formation is apparent at fluoride levels which do not markedly reduce oxygen uptake. Furthermore the decrease in ammonia utilization closely parallels the decrease in amide formation; conversion of ammonia to α-amino acid nitrogen is not so sensitive to fluoride as is glutamine synthesis. It is probable that fluoride acts specifically on some step in the actual combination of glutamate and ammonia.

TABLE V

Fluoride Inhibition of Glutamine Synthesis

The conditions were those of the standard system except for the addition of NaF. 80 mg. of liver in a final volume of 2.0 ml.; reaction period 60 minutes. The data are given in micromoles.

Concentration of NaF	Oxygen used	Ammonia used	Amide formed
M			
0.0	20.2	33.0	18.8
0.0001	19.3	32.4	16.9
0.0003	18.7	29.8	14.5
0.001	18.3	23.4	9.9
0.003	16.4	16.3	3.7
0.01	14.8	12.0	0.7
0.03	10.4	11.0	0.2

Crystal violet has been reported to inhibit the glutamate metabolism of Gram-positive cocci (24) and the synthesis of glutamine in extracts of *Staphylococcus aureus* (25). Low levels of this dye inhibit glutamine synthesis in fresh pigeon liver dispersions. This effect can be observed at a concentration of 0.00001 M, and inhibition is complete at 0.0001 M. Oxygen uptake is also strongly depressed by crystal violet, though not so much as is amide formation. The action of the dye may therefore be to interfere with the supply of oxidative energy rather than to block specifically the linking of glutamate and ammonia.

DISCUSSION

The essential components of the reaction system for glutamine synthesis by pigeon liver dispersions are glutamate, ammonia, phosphate and magnesium ions, and oxygen. The other additions, such as KCl, diphosphopyridine nucleotide, cytochrome *c*, and citrate, probably have only supplementary functions. These results support the view that the energy required for combination of glutamate and ammonia is supplied by the respiration of the liver dispersion. From current knowledge of the manner in which energy is obtained from respiratory processes, it might be anticipated that phosphorylated compounds are involved in glutamine formation. The observation of Krebs (3) that in retina glycolysis can support amide synthesis indicates particularly the participation of adenosine triphosphate. In the case of fresh pigeon liver dispersions, addition of adenosine triphosphate is not essential for glutamine formation but under suitable conditions may accelerate the reaction. When no adenosine triphosphate is added, the small amount present in the liver dispersion may support amide formation by undergoing a rapid cycle of utilization for glutamine synthesis and regeneration by respiration.

Bujard and Leuthardt (26) have recently found that glutamine is formed in rat and guinea pig liver homogenates and that the rate of reaction is greatly accelerated by simultaneous addition of magnesium ions and adenosine triphosphate. In their system the amount of adenosine triphosphate in the tissue homogenate is inadequate to maintain amide formation at nearly maximum rate. The reason for this difference from the pigeon liver system is not clear.

A more adequate picture of the mechanism of glutamine synthesis has been obtained from experiments with tissue extracts, described in the following paper (5). Extracts of acetone-dried pigeon liver catalyze a reaction between glutamate, ammonia, and adenosine triphosphate, in the presence of magnesium ions, to form glutamine, adenosine diphosphate, and inorganic phosphate. This observation confirms the tentative conclusion drawn from studies with tissue slices and homogenates.

SUMMARY

Dilute dispersions of fresh pigeon liver form glutamine when glutamate, ammonia, phosphate and magnesium ions, and oxygen are present. Potassium ions, diphosphopyridine nucleotide, cytochrome *c*, citrate, and other oxidizable substrates, and, under some conditions, adenosine triphosphate and adenosine-5-phosphate accelerate the synthesis. Fluoride ion strongly inhibits the reaction. It is concluded that high energy phosphate compounds formed by respiration, such as adenosine triphosphate, are utilized in linking glutamate and ammonia.

It is a pleasure to acknowledge the assistance of Mrs. Mary Bandurski and Mrs. Anna-May McCreedy in performing these experiments.

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THE ENZYMATIC SYNTHESIS OF GLUTAMINE, A REACTION UTILIZING ADENOSINE TRIPHOSPHATE*

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The preceding paper (2) describes experiments on the synthesis of glutamine in pigeon liver dispersions, which, together with the results of Krebs and his collaborators (3, 4) and of Bujard and Leuthardt (5), suggested that adenosine triphosphate may play an essential rôle in amide formation. Proof for this function of adenosine triphosphate has been obtained in studies with tissue extracts, which are the subject of the present paper. Partially fractionated extracts of acetone-dried pigeon liver have been found to catalyze a stoichiometric reaction between adenosine triphosphate, glutamate, and ammonia, in the presence of magnesium ions, to form glutamine, adenosine diphosphate, and inorganic phosphate.

Methods

Materials—Commercial preparations of most amino acids, amines, and carboxylic acids were used. Diphenylamidophosphate was prepared according to Audrieth and Toy (6) and hydrolyzed to potassium acid amidophosphate by the method of Stokes (7). Methionine sulfoxide was made from DL-methionine by the procedure of Toennies and Kolb (8) as modified by Waelsch *et al.* (9). Adenosine triphosphate was isolated from rabbit muscle by the method of LePage (10); the preparation was carried through two mercury precipitations and finally precipitated as the barium salt with $BaCl_2$. Dried *in vacuo* over $CaCl_2$, the product gave the following analysis: 965 mg. of dibarium adenosine triphosphate contained 5.00 milliatoms of total nitrogen, 1.03 mM of adenine (from measurement of light absorption at 260 mμ), 3.00 mM of total organic phosphate, 1.96 mM of easily hydrolyzable phosphate, and 0.03 mM of inorganic phosphate. Solutions were prepared by dissolving the dibarium salt in dilute HCl, adding a small excess of Na_2SO_4 solution, neutralizing with NaOH to brom thymol blue as an internal indicator, diluting to the desired volume, and centrifuging. Adenosine diphosphate was prepared from adenosine triphosphate by means of yeast hexokinase (11); it contained easily hydrolyzable phos-

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phate equal to 51 per cent of the total organic phosphate. Other materials used are described in the preceding paper (2).

Procedure for Enzyme Experiments—The reaction mixtures were made up in 12 ml. conical tipped centrifuge tubes. The final volume was generally 1.0 or 1.5 ml. for experiments with hydroxylamine and 2 ml. for experiments with ammonia. After all the components except adenosine triphosphate had been added, the samples were placed in a water bath at 30° for 5 minutes; then adenosine triphosphate was added to start the reaction. At the end of the incubation period, the reaction was stopped by addition of an equal volume of 10 per cent trichloroacetic acid, and analyses were performed on the supernatants after centrifugation. Samples identical in essential respects with the incubated samples were treated with trichloroacetic acid before addition of adenosine triphosphate and analyzed for initial values. The changes resulting from enzyme action were obtained by difference.

Analytical Methods—Procedures for the distillation and colorimetric determination of ammonia and for the hydrolysis of glutamine are presented in the preceding paper (2). For inorganic phosphate analyses the method of Gomori (12) was modified by substituting an acid-molybdate reagent containing 5.0 N H_2SO_4 for the original reagent containing 2.5 N H_2SO_4; this decreases the possibility of interference from traces of silicate. Protein was determined by the biuret procedure of Robinson and Hogden (13); a liver protein fraction, the nitrogen content of which had been established by micro-Kjeldahl analysis, was used as a standard.

Hydroxamic acid was determined by a method essentially identical with that of Lipmann and Tuttle (14). The total sample plus an equal volume of 10 per cent trichloroacetic acid or an aliquot of a trichloroacetic acid filtrate was brought to 3.0 ml. with water. 1.0 ml. of 2 M NH_2OH of pH 6.5 ($NH_2OH \cdot HCl$ neutralized with NaOH) and 2.0 ml. of 2.5 per cent $FeCl_3 \cdot 6H_2O$ in 1.5 N HCl were added. If the protein had not been removed previously, the samples were centrifuged, and the colors were read in an Evelyn photoelectric colorimeter with Filter 540, in 18 × 150 mm. tubes. A blank and standards of 1.25 to 3.75 μM of succinyl hydroxamic acid, all containing the same amount of trichloroacetic acid as the unknowns, were read at the same time. Values for hydroxamic acid are expressed as succinyl hydroxamic acid equivalents, since a suitable standard of glutamyl hydroxamic acid was not available.

Results

All the detailed experiments on glutamine synthesis were performed with enzyme preparations obtained from acetone-dried pigeon liver, but similar enzyme activity is found in other tissues and other species of animals. The pigeon liver preparations catalyze a reaction between adenosine triphosphate, glutamate, and ammonia to form glutamine, adenosine diphosphate, and inorganic phosphate. The reaction can be followed by measuring the disappearance of ammonia and formation of amide and inorganic phosphate. Ammonia may be replaced by certain simple derivatives, such as hydroxylamine, hydrazine, and methylamine. When hydroxylamine is substituted for ammonia, a hydroxamic acid is produced. Because of the convenience of analyzing for the hydroxamic acid, hydroxylamine was generally employed in routine enzyme experiments, such as establishment of optimal reaction conditions and assay of fractions during enzyme purification.

Purification and Properties of Pigeon Liver Enzyme—Acetone-dried pigeon liver served as the source of the enzyme preparations. This material was obtained by dispersing fresh pigeon livers in about 6 parts of ice-cold acetone in a Waring blendor, filtering on a Büchner funnel, resuspending the solid in cold acetone, filtering again, and drying on the funnel in a stream of air and then in a vacuum desiccator over $CaCl_2$. Acetone powders kept in a desiccator in the cold yielded active enzyme preparations after at least a year.

Enzymatic activity of acetone powder extracts or of protein fractions obtained from the extracts was assayed in a system of the following composition: 0.1 M tris(hydroxymethyl)aminomethane buffer of pH 7.0 (15), 0.01 M $MgSO_4$, 0.04 M HCN of pH 7 (NaCN freshly neutralized with HCl), 0.05 M sodium L-glutamate, 0.05 M NH_2OH of pH 7 ($NH_2OH \cdot HCl$ freshly neutralized with NaOH), and 0.006 M adenosine triphosphate. The temperature was 30° and the reaction period 10 minutes. About 0.2 ml. of the crude extracts and smaller volumes of the purified fractions were used in each 1 ml. final volume of reaction mixture. Formation of hydroxamic acid and inorganic phosphate was measured. The same protein fractions were incubated in a system containing only buffer, magnesium ions, cyanide, and adenosine triphosphate; liberation of inorganic phosphate in this system was used as a measure of hydrolysis of adenosine triphosphate.

A study of conditions for extraction of the enzyme activity from the acetone powder showed that alkaline salt solutions were most effective, and 0.2 M $NaHCO_3$ was selected as the best of the solutions tested. Generally the acetone powder was ground with 10 parts of ice-cold 0.2 M $NaHCO_3$, and the solid was removed by centrifugation after the mixture had stood in the ice box for a few hours. Reextraction of the solid with 5 parts of 0.2 M $NaHCO_3$ increased the yield of activity by about one-third.

Extracts of acetone-dried pigeon liver cause hydrolysis of adenosine triphosphate. This activity interferes with the study of glutamine synthesis by obscuring the stoichiometry and diminishing the quantity of adenosine triphosphate available for the synthesis. Hydrolysis of adenosine triphosphate by pigeon liver extracts is strongly activated by magnesium ions; calcium ions are less effective. Different batches of acetone powder yield different levels of adenosinetriphosphatase activity. Extracts from one powder, which was used for the experiment described in a previous note (1), contained little adenosinetriphosphatase and gave fairly good stoichiometry in glutamine formation without purification. Extracts from another large batch of powder, which was used in all the experiments described in the present paper, brought about hydrolysis of adenosine triphosphate almost as rapidly as utilization for glutamine synthesis. The extracts did not show significant glutaminase activity.

TABLE I

Enzyme Assays during Purification

The fractionation procedure and assay systems are described in the text. Hydroxamic acid and phosphatase activities are expressed as micromoles of hydroxamic acid or phosphate per ml. of enzyme solution per 10 minutes and protein as mg. of N per ml. of enzyme solution.

Fraction	Volume	Hydroxamic acid activity		Phosphatase activity	Phosphatase / Hydroxamic acid	Protein	Hydroxamic acid / Protein
		Concentration	Total				
	ml.	*μM per ml.*	*μM*	*μM per ml.*		*mg. N per ml.*	*μM per mg. N*
Extract	246	8.3	2040	5.2	0.63	4.78	1.74
Isoelectric ppt.	120	16.5	1980	2.4	0.15	2.80	5.90
Ppt. 22% ethanol	35	10.0	350	3.5	0.35	3.72	2.68
" 22-43% ethanol	35	20.2	710	0.0	0.00	1.09	18.5

Studies on the precipitation of enzyme activity were carried out so that a method for freeing the extracts from adenosinetriphosphatase might be devised. A purification procedure was adopted which consisted of isoelectric precipitation and ethanol fractionation. All operations were carried out with cold solutions in a room at a temperature of about 1°. One such preparation is described here, and the assay results are given in Table I. (*a*) 20 gm. of pigeon liver acetone powder were extracted twice, first with 180 ml. and then with 100 ml. of 0.2 M $NaHCO_3$. The combined solutions were dialyzed overnight against 0.15 M NaCl-0.005 M $NaHCO_3$ and assayed (extract). (*b*) 246 ml. of extract were mixed with 123 ml. of 0.2 M acetate of pH 4.2. The precipitate was removed by centrifugation and suspended in a mixture of 50 ml. of 0.2 M $NaHCO_3$ and 30 ml. of 0.15 M NaCl-0.005 M $NaHCO_3$. After an hour the solid was centrifuged and reextracted with

40 ml. of NaCl-$NaHCO_3$. The combined solutions were dialyzed overnight against NaCl-$NaHCO_3$ and assayed (isoelectric precipitate). (*c*) To 120 ml. of the solution of the isoelectric precipitate 34 ml. of ethanol were slowly added, with efficient stirring and cooling in an ice bath; the final ethanol concentration was 22 volumes per cent. After 15 minutes the precipitate was centrifuged and suspended in 35 ml. of NaCl-$NaHCO_3$, and the solution was clarified by centrifugation (precipitate, 22 per cent ethanol). (*d*) 136 ml. of supernatant from the first ethanol precipitation were treated in the same manner with a further 50 ml. of ethanol, to give a final ethanol concentration of 43 volumes per cent. The precipitate was centrifuged and dissolved in NaCl-$NaHCO_3$, and the slight turbidity was removed by centrifugation (precipitate, 22 to 43 per cent ethanol).

It can be seen from the data of Table I that 35 per cent of the original activity was obtained in the fraction precipitating between 22 and 43 volumes per cent of ethanol; this fraction has an activity per unit protein 10 times as great as that of the original extract and is free from adenosinetriphosphatase activity. This degree of purification is consistently achieved, and the yield of activity is usually greater than in this particular example (up to 50 per cent of the original activity). Different preparations of this fraction were used for the experiments with the pigeon liver enzyme which are reported in this paper. A repetition of the isoelectric precipitation and ethanol fractionation has given a preparation 20 times as active per unit protein as the original extract.

The enzyme activity in both the original dialyzed extracts and the purified fractions is quite stable. The preparations may be kept at 2° for several weeks without significant loss in activity. The activity of the original extracts is not diminished by incubation for 3 hours at 30°. The heat stability of the enzyme in the crude extracts was tested by heating for 5 minutes at various temperatures in the presence of 0.05 M L-glutamate, which was added to protect specifically the glutamine enzyme. No activity was lost at temperatures up to 50°, but complete inactivation occurred at 60°. No differential effect on glutamine synthesis and adenosinetriphosphatase activity was observed.

The enzyme is associated with a protein fraction having the solubility properties of a euglobulin. When crude extracts or purified fractions are thoroughly dialyzed against distilled water, a precipitate appears which contains all the enzyme activity and which can be redissolved in 0.15 M NaCl-0.005 M $NaHCO_3$. Nearly all the protein of the purified fraction is precipitated by water dialysis.

It is not yet clear whether the pigeon liver preparations contain one or more than one protein component essential for the over-all enzymatic reaction.

Stoichiometry of Reaction—The formation of amide by pigeon liver preparations requires the simultaneous presence of glutamate, amine, adenosine triphosphate, and magnesium ions. Omission of any one of these components leads to complete inactivity in the synthesis. The stoichiometry is most clearly seen in experiments with ammonia as the amine, when measurements of disappearance of ammonia, formation of amide, and liberation of inorganic phosphate can be made. The amide is determined as ammonia released by heating with 1 N H_2SO_4 for 11 minutes at 100°, after previous removal of free ammonia by vacuum distillation. Table II gives the results of an experiment showing parallel utilization of ammonia and formation of amide and inorganic orthophosphate during various reaction periods. A series of 52 individual samples, similar to those described in Table II but including variations in pH, in quantity of enzyme, and in concentration of adenosine triphosphate and ammonia, gave the following average ratios: 1.05 moles of ammonia used, 1.00 mole of amide formed, and 1.00 mole of inorganic phosphate liberated. In the individual samples amide and phosphate were always formed in proportions very close to 1:1, but ammonia utilization was more variable, probably because it was determined less accurately as the difference between large initial and final ammonia values.

TABLE II

Stoichiometry of Glutamine Synthesis

The samples contained 0.1 M tris(hydroxymethyl)aminomethane buffer of pH 7.55, 0.01 M $MgSO_4$, 0.04 M HCN of pH 7.4, 0.05 M L-glutamate, 0.01 M NH_4Cl, 0.006 M adenosine triphosphate, and 0.05 ml. of purified pigeon liver enzyme (31 γ of N) in 1.0 ml. Temperature 30°; times as indicated. The figures given are micromoles per ml. of reaction mixture.

Time	Ammonia used	Glutamine formed	Phosphate liberated
min.			
5	1.7	1.94	1.80
10	3.1	2.98	2.96
20	3.9	4.08	3.94
30	4.2	4.72	4.83

These results indicate the formation of a monoamide of glutamic acid. This amide is very labile and is completely split by acid under the same conditions which give quantitative results with authentic samples of glutamine, the γ-amide of glutamic acid. The observations of Melville (16) show that the α-amide, isoglutamine, is much more stable and is comparable to asparagine, which requires 3 hours for complete hydrolysis by 1 N

H_2SO_4 at 100° (17). The formation of glutamine by pigeon liver is known from the work of Örström *et al.* (4), who isolated in crystalline form an amide produced by pigeon liver slices from pyruvate and ammonia and identified it as glutamine hydrochloride. It may be concluded that the amide formed in the present experiments is glutamine.

Two observations indicate that adenosine diphosphate is the end-product formed from adenosine triphosphate. First, when samples containing relatively large amounts of enzyme are incubated for long periods of time, the reaction stops when 1 mole of glutamine and 1 mole of inorganic phosphate have been formed for each mole of adenosine triphosphate added. Second, adenosine diphosphate itself cannot be utilized in the test system, unless the enzyme myokinase is added to convert it in part to adenosine triphosphate.

On the basis of these findings the reaction may be written as shown in Equation 1.

$$\text{Glutamate} + \text{ammonia} + \text{adenosine triphosphate} \rightarrow \text{glutamine} + \text{adenosine diphosphate} + \text{phosphate} \quad (1)$$

When hydroxylamine is substituted for ammonia, a hydroxamic acid is formed, which can be determined colorimetrically by reaction with $FeCl_3$, and at the same time phosphate is liberated from adenosine triphosphate. Data from several individual experiments are presented later, and only average values are given here. A series of 154 samples, similar in composition to the reaction system for enzyme assay but including variations of reaction time, enzyme level, pH, and concentration of each of the reactants, gave the following relation: for 1.00 mole of inorganic phosphate released, 1.12 moles of succinyl hydroxamic acid equivalent were formed. The proportion was not always constant, varying from 0.98 to 1.80 as extremes; this was due in part to variability in the hydroxamic acid determination. The changes were not related to alterations in the conditions of the enzyme reaction. The failure to observe a 1:1 relation between phosphate liberation and hydroxamic acid formation probably results from the use of a standard of succinyl hydroxamic acid, which may not give the same color per mole as the hydroxamic acid formed from glutamate.

Reaction between adenosine triphosphate, glutamate, and ammonia at neutral pH should result in the formation of H^+ ions. For purposes of calculation the over-all process may be divided into two steps: (1) combination of carboxylation ion and ammonium ion (Equation 2),[1]

$$R_1COO^- + NH_4^+ \rightarrow R_1CONH_2 + H_2O \quad (2)$$

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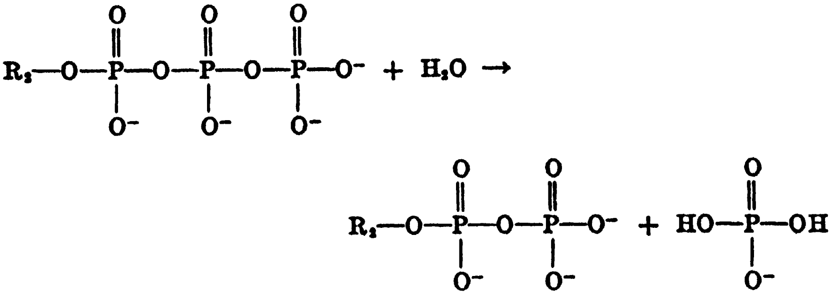
[1] R_1 represents $COO^-CHNH_3^+CH_2CH_2^-$.

which involves no acid-base change, and (2) splitting of inorganic phosphate from adenosine triphosphate (Equation 3),[3]



$$R_2{-}O{-}\underset{O^-}{\overset{O}{\overset{\|}{\underset{|}{P}}}}{-}O{-}\underset{O^-}{\overset{O}{\overset{\|}{\underset{|}{P}}}}{-}O{-}\underset{O^-}{\overset{O}{\overset{\|}{\underset{|}{P}}}}{-}O^- + H_2O \rightarrow R_2{-}O{-}\underset{O^-}{\overset{O}{\overset{\|}{\underset{|}{P}}}}{-}O{-}\underset{O^-}{\overset{O}{\overset{\|}{\underset{|}{P}}}}{-}O^- + HO{-}\underset{O^-}{\overset{O}{\overset{\|}{\underset{|}{P}}}}{-}OH \qquad (3)$$

which results in the formation of one acid group (inorganic phosphate) with a pK′ of 6.8. Therefore, at pH 7.4, for each mole of glutamine (or phosphate) formed, 0.80 mole of H^+ ion should be released. When hydroxylamine, with a pK′ of about 5.9, replaces ammonia, the first step becomes (Equation 4)

$$R_1COO^- + NH_2OH \rightarrow RCONHOH + OH^- \qquad (4)$$

It can be calculated that 0.97 mole of OH^- ion should be released in this step; so that the over-all acid-base change in hydroxamic acid formation at pH 7.4 should be liberation of 0.17 mole of OH^- ion. In the same way it can be estimated that reaction with methylamine, pK 10.6, should release 0.80 mole of H^+ ion, as in the case of ammonia, and that reaction with hydrazine, pK 8.5, should release 0.73 mole of H^+ ion. Table III gives the results of an experiment with different amines in bicarbonate buffer. Acid-base changes were followed by measuring manometrically the CO_2 released or absorbed, and formation of inorganic phosphate in the same samples was used as a measure of amide synthesis. The observed evolution of CO_2 is in rather good agreement with that calculated by multiplying the phosphate by the factors described above.

Intermediates—Since it seemed probable that a process involving so many components as glutamine synthesis proceeds in steps, attempts were made to detect intermediate compounds.

Reaction of adenosine triphosphate with glutamate might produce such compounds as γ-glutamyl phosphate or γ-glutamyl adenosine diphosphate, which then could react with an amine to yield the amide. It might be expected that enzymatic catalysis would be necessary for the first step and perhaps also for the second. To test for the formation of an intermediate from glutamate, reaction mixtures containing buffer, adenosine triphosphate, glutamate, Mg^{++} ions, cyanide, and purified pigeon liver enzyme were incubated for various times, and the enzyme was then inactivated by mild treatment so as to preserve labile compounds. Heating for 30 seconds in boiling water and immediately cooling and treating with ice-cold trichloroacetic acid and immediately neutralizing were two methods used; a substance like acetyl phosphate would suffer relatively little decomposition during such procedures. The samples were then analyzed for acyl phosphates by the hydroxamic acid method of Lipmann and Tuttle (14) (an acyl adenosine diphosphate might also react in this determination) and by differential phosphate determinations according to Lowry and Lopez (18) and Gomori (12). No intermediate was detected. It may be mentioned that if the enzyme was not inactivated before the addition of neutral hydroxylamine, as in the original method of Lipmann and Tuttle (14), hydroxamic acid was always found. Since the pigeon liver enzyme remains completely active in the presence of high concentrations of hydroxylamine, hydroxamic acid formation under these conditions cannot be regarded as evidence for the enzymatic synthesis and accumulation of an acyl phosphate which subsequently reacts non-enzymatically with hydroxylamine.

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[3] R_2OH represents adenosine.

TABLE III

Acid Formation during Amide Synthesis

The samples were made up in Warburg vessels and contained 0.03 M $NaHCO_3$, 0.01 M $MgSO_4$, 0.04 M HCN of pH 7.4, 0.05 M L-glutamate, 0.02 M amine, 0.006 M adenosine triphosphate (tipped in from the side arm after temperature equilibration), and 0.10 ml. of purified pigeon liver enzyme (160 γ of N) in 2.0 ml. Gas phase 5 per cent CO_2-95 per cent N_2; temperature 30°; time 30 minutes. The figures given are micromoles per ml. of reaction mixture. Calculated CO_2 evolution was obtained by multiplying the phosphate formed by the factors given in the text.

Amine	Phosphate liberated	CO_2 evolution calculated	CO_2 evolution observed
None	0.05	0.04	0.2
Ammonium chloride	4.1	3.3	3.1
Hydroxylamine	3.4	−0.6	0.4
Hydrazine hydrochloride	3.2	2.3	2.3
Methylamine hydrochloride	2.3	1.8	2.0

Adenosine triphosphate might yield phosphate or adenosine diphosphate derivatives of the amines, which then could react with glutamate to give the corresponding amides. To test this possibility, reaction mixtures containing buffer, adenosine triphosphate, hydroxylamine, Mg^{++} ions, cyanide, and enzyme were incubated for various times, and the enzyme was then inactivated. Differential phosphate analyses failed to show accumulation of labile phosphate compounds. Addition of glutamate did not result in hydroxamic acid formation; this indicates that intermediates capable of reacting non-enzymatically with glutamate were not present. One intermediate which might be formed from adenosine triphosphate and ammonia, amidophosphate, was prepared synthetically and tested with the purified pigeon liver enzyme. Amidophosphate could not replace adenosine triphosphate plus ammonia in reacting with glutamate to form glutamine. It was slowly hydrolyzed by the enzyme preparations and could partially replace ammonia, provided adenosine triphosphate was also added. This lack of reactivity indicates that amidophosphate is probably not an intermediate in glutamine synthesis.

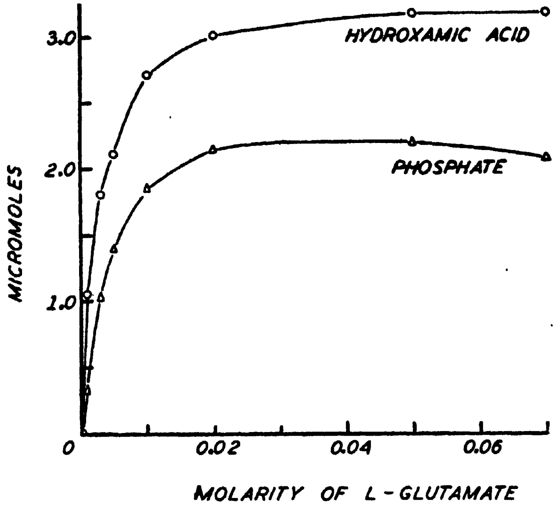

FIG. 1. Effect of glutamate concentration on the rate of amide synthesis and phosphate liberation. The samples contained 0.01 M $MgSO_4$, 0.04 M HCN of pH 7, 0.4 M NH_2OH of pH 7.0, L-glutamate in the concentrations shown, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°; time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

These negative results are not conclusive evidence for a reaction mechanism involving only one step or against a mechanism involving two or more steps. The experimental procedures available were not completely satisfactory for detecting the types of intermediates suggested above. If intermediates exist, they may be exceedingly labile or may not accumulate in the incomplete systems in sufficient quantities for analysis.

The following sections describe the effects of varying the conditions of reaction and the components of the system.

Glutamate—In Fig. 1 the rate of amide synthesis is plotted as a function of the level of L-glutamate. A glutamate concentration of 0.05 M is required to give a maximum rate. The method of Lineweaver and Burk (19) was used in calculating the glutamate-enzyme dissociation constant from such data. Data from three experiments gave an average value for the dissociation constant of 0.0027 M (range 0.0011 to 0.0038 M).

A number of other carboxylic acids were tested for their ability to replace glutamate in forming a hydroxamic acid, but none were effective, either in the original dialyzed extracts or in the purified fraction. The substances tested included formate, acetate, butyrate, succinate, fumarate, *cis*-aconitate, lactate, malate, citrate, pyruvate, oxalacetate, α-ketoglutarate, glycine, alanine, valine, leucine, isoleucine, serine, threonine, hydroxyproline, cysteine, methionine, aspartate, phenylalanine, tyrosine, tryptophan, histidine, arginine, lysine, and *p*-aminobenzoate.

TABLE IV

Activity of Various Amines in Amide Synthesis

The samples contained 0.1 M tris(hydroxymethyl)aminomethane buffer of pH 7.0, 0.01 M $MgSO_4$, 0.04 M HCN of pH 7, 0.05 M L-glutamate, 0.02 M amine, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°; time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

System	Phosphate liberated
No glutamate or amine	0.06
With glutamate, no amine	0.18
Glutamate + methylamine hydrochloride	2.02
" + ammonium chloride	2.23
" + hydroxylamine	2.28
" + hydrazine hydrochloride	2.60
" + L-cysteine	0.78

Amine—The ability of various amines to react with glutamate plus adenosine triphosphate was tested by taking advantage of the fact that in the absence of amines there occurs virtually no breakdown of adenosine triphosphate, while in the presence of a reactive amine inorganic phosphate is liberated. Ammonia, hydroxylamine, hydrazine, and methylamine were found to react at approximately the same rates (Table IV) and cysteine more slowly. The following amines and amino acids gave negative results by this testing procedure: dimethylamine, trimethylamine, *p*-aminobenzoate, glycine, alanine, valine, leucine, isoleucine, serine, threonine, hydroxyproline, methionine, aspartate, phenylalanine, tyrosine, tryptophan, histidine, arginine, and lysine.

It has previously been indicated that the reaction product formed with ammonia is glutamine, the γ-amide of glutamic acid. Hydroxylamine yields a substance giving a violet color with $FeCl_3$, characteristic of a hydroxamic acid, an N-substituted amide. It is very probable that all the amines effective in the testing procedure described above react in the same way, and this supposition is supported by the observation that the other amines are competitive with respect to hydroxylamine. When added along with hydroxylamine, they depress hydroxamic acid formation, while total amide synthesis as measured by phosphate liberation proceeds at a rate intermediate between those observed with the two amines separately (Table V).

TABLE V

Competition of Amines in Amide Synthesis

The samples contained 0.1 M tris(hydroxymethyl)aminomethane buffer of pH 7.0, 0.01 M $MgSO_4$, 0.04 M HCN of pH 7, 0.05 M L-glutamate, 0.02 M amine, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme (150 γ of N) in 1.0 ml. Temperature 30°, time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

Amine	Hydroxamic acid formed	Phosphate liberated
Hydroxylamine	3.20	2.78
Ammonia		3.28
Hydrazine		3.76
Methylamine		2.18
Hydroxylamine + ammonia	1.09	3.16
" + hydrazine	1.75	3.58
" + methylamine	2.67	2.89

TABLE VI

Effect of Ammonia Concentration on Glutamine Synthesis

The samples contained 0.1 M tris(hydroxymethyl)aminomethane buffer of pH 7.5, 0.01 M $MgSO_4$, 0.04 M HCN of pH 7.5, 0.05 M L-glutamate, NH_4Cl as indicated, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°; time 20 minutes. The figures given are micromoles per ml. of reaction mixture.

NH_4Cl added	Glutamine formed	Phosphate liberated
0.0	0.27	0.14
1.0	1.45	1.20
3.0	3.08	3.20
5.0	5.11	5.04
10.0	5.25	5.34
20.0	5.02	5.07

The dissociation constants for reversible combination of ammonia or hydroxylamine with the enzyme are probably quite low. When the concentration of amine is decreased, the rate of reaction is not diminished until the absolute quantity of amine limits stoichiometrically the extent of reaction. Illustrative data for ammonia are given in Table VI.

Adenosine Triphosphate—Curves showing the relation between rate of amide synthesis and adenosine triphosphate concentration are given in Fig. 2. No amide is formed when adenosine triphosphate is omitted.

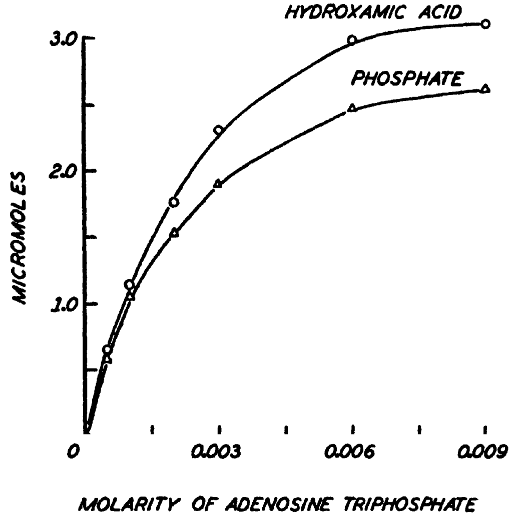

FIG. 2. Effect of adenosine triphosphate concentration on the rate of amide synthesis and phosphate liberation. The samples contained 0.01 M $MgSO_4$, 0.04 M HCN of pH 7, 0.05 M L-glutamate, 0.4 M NH_2OH of pH 7.0, adenosine triphosphate in the concentrations shown, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°, time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

Application of the method of Lineweaver and Burk (19) to such data gave an average value for the adenosine triphosphate-enzyme dissociation constant of 0.0025 M (three experiments, range 0.0020 to 0.0028 M). It is not practical in routine experiments to add sufficient adenosine triphosphate to saturate the enzyme (a concentration of at least 0.02 M would be required); instead the initial concentration of adenosine triphosphate was kept uniform in samples which were to be compared.

Adenosine-5-monophosphate and adenosine diphosphate cannot replace adenosine triphosphate in the usual test system, but adenosine diphosphate is active if the enzyme myokinase is added to convert it to adenosine triphosphate plus adenosine monophosphate (Table VII). Both adenosine diphosphate and adenosine monophosphate inhibit amide synthesis when they are present along with adenosine triphosphate. Since it seemed likely that this represented a competitive inhibition, rates of reaction were determined for samples containing various concentrations of adenosine triphosphate in the presence and absence of different concentrations of adenosine monophosphate and diphosphate, and the data were analyzed according to Lineweaver and Burk (19). The results agreed with the hypothesis of competitive inhibition, and the dissociation constants for both adenosine monophosphate and adenosine diphosphate and the enzyme were estimated to be about 0.01 M.

TABLE VII

Effect of Adenosine Monophosphate and Adenosine Diphosphate on Amide Synthesis

The samples contained 0.01 M $MgSO_4$, 0.04 M HCN of pH 7, 0.05 M L-glutamate, 0.4 M NH_2OH of pH 7.0, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Adenine nucleotides were added in a concentration of 0.006 M. Temperature 30°; time 10 minutes. The figures given are micromoles per ml. of reaction mixture. Myokinase was prepared according to Colowick and Kalckar (11) and lyophilized.

Addition	Hydroxamic acid formed	Phosphate liberated
No nucleotide	0.03	0.07
Adenosine-5-monophosphate	0.00	0.14
Adenosine diphosphate	0.27	0.13
" " + 7 mg. myokinase	1.84	1.52
" triphosphate	3.31	2.28
" " + adenosine monophosphate	2.74	2.16
" " + " diphosphate	2.54	1.51

Magnesium Ions—Addition of Mg^{++} ions is essential for amide synthesis. Variation of reaction rate with Mg^{++} concentration is shown in Fig. 3; a maximum rate is obtained with 0.01 M $MgSO_4$. The curve is of such a shape as to suggest combination of more than one Mg^{++} ion with each molecule of enzyme. Mn^{++} ions can replace Mg^{++} ions; the optimal concentration of Mn^{++} is 0.002 M and the maximum rate is only half that obtained with Mg^{++}.

Reducing Agents—Addition of cysteine, glutathione, or cyanide is necessary to achieve maximum rates of amide formation with crude or purified liver enzyme preparations (Table VIII). Probably free sulfhydryl groups on the enzyme protein are essential for activity. Cyanide gives the most rapid reaction rates, and a concentration of 0.04 M is sufficient to produce a maximum effect. When a reducing agent is present, the reaction rate is the same with either air or nitrogen in the gas phase. In performing all enzyme experiments, the enzyme and reducing agent were mixed in an incomplete reaction system which was incubated for 5 minutes before adenosine triphosphate was added to start amide synthesis.

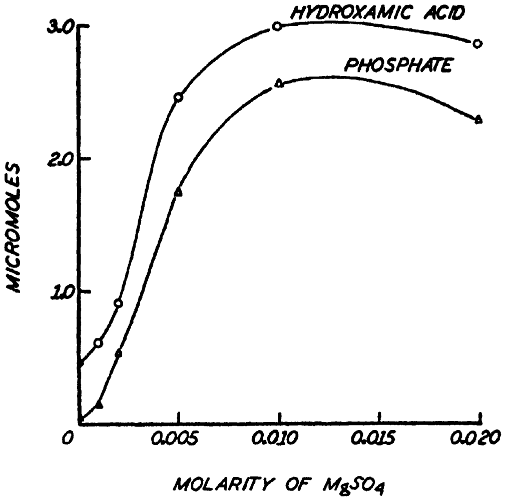

FIG. 3. Effect of Mg^{++} concentration on the rate of amide synthesis and phosphate liberation. The samples contained $MgSO_4$ in the concentrations shown, 0.04 M HCN of pH 7, 0.05 M L-glutamate, 0.4 M NH_2OH of pH 7.0, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°; time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

TABLE VIII

Effect of Reducing Agents on Amide Synthesis

The samples contained 0.01 M $MgSO_4$, 0.05 M L-glutamate, 0.4 M NH_2OH of pH 7.0, 0.006 M adenosine triphosphate, reducing agents in a concentration of 0.04 M, and 0.13 ml. of purified pigeon liver enzyme (81 γ of N) in 1.0 ml. Gas phase air; temperature 30°; time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

Reducing agent	Hydroxamic acid formed	Phosphate liberated
None	2.31	1.93
HCN	3.42	2.86
Cysteine	2.96	2.56
Glutathione	2.86	2.49

pH—If hydroxylamine is added as the amine component and hydroxamic acid formation is measured, the reaction occurs most rapidly when the pH of the buffer is 7.0 (Fig. 4). The rate falls off rapidly on the acid side and more slowly on the alkaline side of this pH. When ammonia is the amine and glutamine synthesis is measured, the optimal pH lies between 7.5 and 8.0. This difference may be related to the difference between the pK′ values of the two bases (5.9 for hydroxylamine and 9.4 for ammonia).

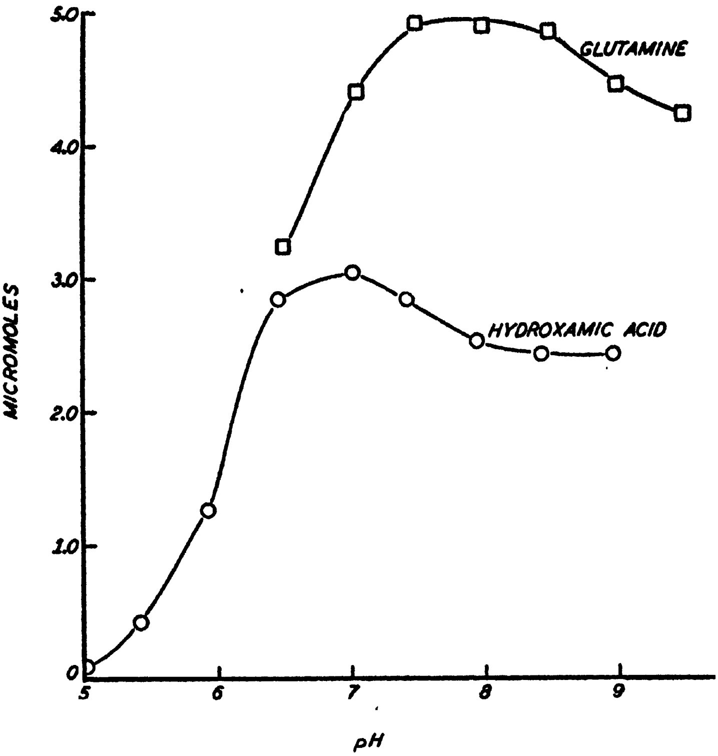

FIG. 4. Effect of pH on the rate of amide synthesis. The hydroxamic acid samples contained 0.01 M $MgSO_4$, 0.04 M HCN, 0.05 M L-glutamate, 0.4 M NH_2OH at the pH shown, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°; time 10 minutes. The glutamine samples contained 0.1 M buffer of the pH shown, 0.01 M $MgSO_4$, 0.04 M HCN, 0.05 M L-glutamate, 0.02 M NH_4Cl, 0.006 M adenosine triphosphate, and 0.10 ml. of purified pigeon liver enzyme in 1.0 ml. Temperature 30°; time 20 minutes. Tris(hydroxymethyl)aminomethane buffer was used up to pH 8 and 2-amino-2-methyl-1,3-propanediol buffer (15) from pH 8.5 to 9.5. The figures given are micromoles per ml. of reaction mixture.

Other Components—It is unlikely that other substances readily dissociable from the enzyme protein are essential for glutamine synthesis by pigeon liver preparations. The enzyme may be dialyzed for long periods of time without inactivation, and during purification large losses of total activity do not occur. In particular the cofactor containing pantothenic acid which is required for acetylation of sulfanilamide (20) and choline (21) is not involved in glutamine formation. Prolonged storage in the ice box or incubation for a few hours at room temperature does not reduce the activity of pigeon liver extracts in synthesizing glutamine, although such treatment completely abolishes the capacity for acetylation of sulfanilamide by causing destruction of the coenzyme (22).[3]

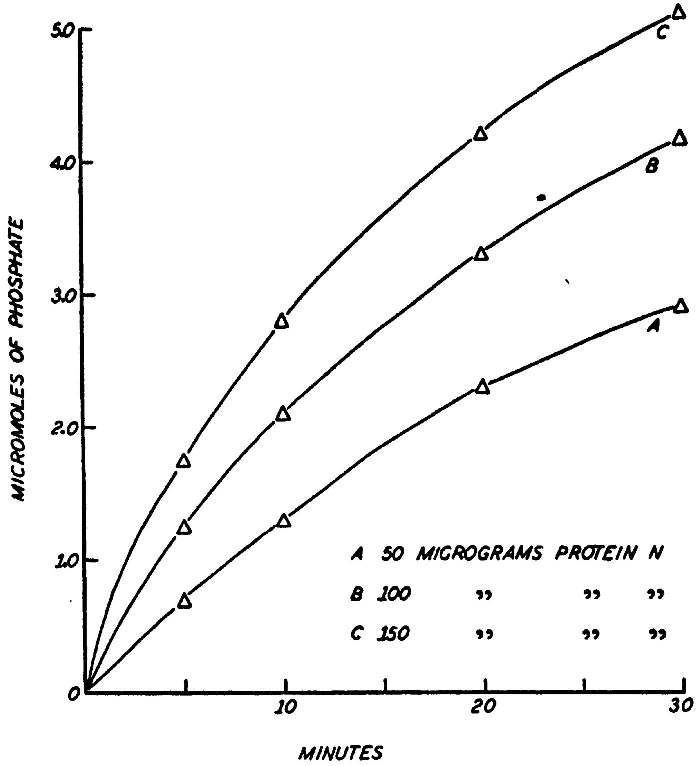

FIG. 5. Kinetics of phosphate liberation during amide synthesis. The samples contained 0.1 M tris(hydroxymethyl)aminomethane buffer of pH 7.0, 0.04 M HCN of pH 7, 0.01 M $MgSO_4$, 0.05 M L-glutamate, 0.05 M NH_2OH of pH 7, 0.006 M adenosine triphosphate, and the indicated quantities of purified pigeon liver enzyme in 1.0 ml. Temperature 30°; times as shown. The figures given are micromoles per ml. of reaction mixture.

Kinetics—The course of amide synthesis over a period of 30 minutes with different quantities of enzyme is represented in Fig. 5. Hydroxylamine was present as the amine, but only data for phosphate liberation from adenosine triphosphate are given. In this test system all the components except adenosine triphosphate are present in sufficient excess to saturate the enzyme protein for the whole reaction period. Calculations indicate that the reaction rate falls off too rapidly to be first order with respect to adenosine triphosphate. By use of the experimentally determined dissociation constants and the concentrations of adenosine diphosphate (equal to the inorganic phosphate liberated) and of adenosine triphosphate (from the initial concentration by difference), the concentration of the adenosine triphosphate-enzyme complex at each time interval can be calculated, but the observed reaction rates decline more rapidly than the concentration of this complex. A simple formulation of the kinetics is not apparent at present.

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[3] In a personal communication Dr. Fritz Lipmann has stated that addition of a purified preparation of his coenzyme A to dialyzed pigeon liver extracts does not accelerate hydroxamic acid formation from glutamate.

TABLE IX

Effect of Sodium Fluoride on Amide Synthesis

The samples contained 0.01 M $MgSO_4$, 0.04 M HCN of pH 7, 0.05 M L-glutamate, 0.2 M NH_2OH of pH 7.0, 0.006 M adenosine triphosphate, sodium fluoride as indicated, and 0.10 ml. of purified pigeon liver enzyme (62 γ of N) in 1.0 ml. Temperature 30°; time 10 minutes. The figures given are micromoles per ml. of reaction mixture.

NaF added	Hydroxamic acid formed	Phosphate liberated
M		
0.0	3.94	3.30
0.0001	3.55	2.73
0.0003	2.61	2.22
0.001	1.55	1.07
0.003	0.64	0.35
0.01	0.39	0.06
0.03	0.42	0.07

The variation of reaction rate with temperature was studied over the range 10–40°, with phosphate liberation in a system containing hydroxylamine as a measure of rate. The following temperature coefficients (Q_{10}) were observed: 3.1 for the temperature interval 10–20°, 2.1 for the interval 20–30°, and 1.8 for the interval 30–40°.

Inhibitors—The formation of amide by pigeon liver enzyme preparations is strongly depressed by sodium fluoride in low concentrations. Illustrative data are given in Table IX. The inhibition is apparent with 0.0001 M fluoride and is nearly complete with 0.01 M fluoride. The inhibitory action of sodium fluoride on glutamine synthesis in fresh pigeon liver dispersions (2) is probably a result of this specific effect on the linking of glutamate and ammonia.

Methionine sulfoxide was found by Waelsch *et al.* (9) to inhibit the growth of lactobacilli; the inhibition could be overcome by addition of large amounts of glutamate or small amounts of glutamine, and it was suggested that methionine sulfoxide acts as a competitive inhibitor for the formation of glutamine from glutamate. McIlwain *et al.* (23) have shown that methionine sulfoxide prevents formation of glutamine by streptococci. Recently Elliott and Gale (24) have demonstrated directly that methionine sulfoxide is a competitive inhibitor with respect to glutamate for the formation of glutamine from glutamate, ammonia, and adenosine triphosphate in extracts of staphylococci. DL-Methionine sulfoxide also depresses the synthesis of glutamine by purified pigeon liver preparations. Experiments with varying concentrations of glutamate and methionine sulfoxide were run, and analysis of the results by the method of Lineweaver and Burk (19) showed that methionine sulfoxide was competing with glutamate for combination with the enzyme. The effects are much smaller than those observed with the bacterial extracts; with pigeon liver enzyme 0.09 M DL-methionine sulfoxide in the presence of 0.01 M L-glutamate causes 50 per cent inhibition, while with staphylococcus extracts 0.011 M methionine sulfoxide in the presence of 0.033 M L-glutamate causes 72 per cent inhibition. This variation results from differences in the dissociation constants for the glutamate and methionine sulfoxide complexes of the two enzyme proteins. With purified pigeon liver enzyme the dissociation constant for L-glutamate is 0.0027 M and for DL-methionine sulfoxide about 0.03 M. From the graph given by Elliott and Gale (24) the dissociation constants for the staphylococcus enzyme can be estimated as 0.03 M for L-glutamate and 0.002 M for methionine sulfoxide. The lower dissociation constant for methionine sulfoxide and the higher constant for glutamate should make the bacterial enzyme much more susceptible to competitive inhibition than the pigeon liver enzyme.

Crystal violet has been reported to inhibit glutamate metabolism in Gram-positive cocci (25) and glutamine synthesis in extracts of *Staphylococcus aureus* (24), and it was found to depress glutamine formation in fresh pigeon liver dispersions (2). In concentrations up to 0.001 M this dye does not affect the rate of glutamine synthesis in pigeon liver extracts, although such concentrations completely inhibit amide formation in pigeon liver dispersions or bacterial extracts. Probably crystal violet depresses glutamine synthesis in pigeon liver dispersions by interfering with the supply of oxidative energy available through respiration, since oxygen uptake is also inhibited. The difference between extracts of pigeon liver and of bacteria in sensitivity to crystal violet presumably is due to differences in the enzyme proteins, since the reactions catalyzed by the two preparations are the same (adenosine triphosphate, glutamate, and ammonia, reacting in the presence of Mg^{++} ions and reducing agents to give glutamine).

Distribution of Enzyme Activity—Acetone powders of brain, liver, and kidney of various species were extracted with bicarbonate solutions and tested for activity by the hydroxamic acid procedure. Single batches of these powders, sometimes rather old, were used; so that for the most part quantitative comparisons of activity could not be made. Some activity was found in all the acetone powders tested; these included pigeon liver and brain, rat liver, kidney, and brain, guinea pig liver, kidney, and brain, rabbit liver, kidney, and brain, cat liver, kidney, and brain, beef liver, and pig liver. When comparable powders of different tissues from a single species were available, it was found that brain gave more active enzyme preparations than did liver or kidney (except for the pigeon). Pigeon liver powder was the best source of enzyme in terms of total activity per unit weight of powder or of activity per unit of extracted protein. The various acetone powder extracts also caused hydrolysis of adenosine triphosphate, but those preparations which formed hydroxamic acid readily also split inorganic phosphate from adenosine triphosphate more rapidly when the components of the amide-synthesizing system (glutamate plus hydroxylamine) were present than when they were omitted. This observation, together with the fact that no hydroxamic acid was formed when adenosine triphosphate was not added, indicates that the enzymes from other sources catalyze the same sort of reaction between adenosine triphosphate, glutamate, and amine that has been demonstrated for the pigeon liver enzyme.

DISCUSSION

These studies show that the synthesis of glutamine in enzyme preparations from pigeon liver involves a reaction between glutamate, ammonia, and adenosine triphosphate, in the presence of magnesium ions, to form the amide, adenosine diphosphate, and inorganic phosphate. Adenosine triphosphate supplies the energy necessary for the synthesis of the amide linkage by participating stoichiometrically in the reaction. Similar enzyme activity is found in brain, liver, and kidney of a number of vertebrates. Elliott (26) has described a similar reaction system for glutamine synthesis, using acetone-dried sheep brain as the source of enzyme, and Elliott and Gale (24) have obtained extracts of *Staphylococcus aureus* which bring about the same reaction. It is likely that an analogous mechanism is utilized in the synthesis of glutamine and asparagine in plants.

The enzymatic synthesis of glutamine shows interesting similarities to the synthesis of acetylcholine (27, 28) and of sulfanilamide (22, 29). In these reactions adenosine triphosphate is utilized as the source of energy for the formation of an amide or ester linkage. In all cases the enzymes are found in extracts of acetone powders, and reducing agents must be added for maximum activity. However, there are significant differences among these systems. Mg^{++} ions are necessary for the synthesis of glutamine and of acetylcholine (30) but not of acetyl sulfanilamide (22). A cofactor containing pantothenic acid is required for the acetylation reactions (20, 21) but not for glutamine formation.

Utilization of the energy of adenosine triphosphate for the synthesis of amide linkages seems to be a reaction of general importance. In addition to glutamine and acetyl sulfanilamide, *p*-aminohippuric acid (31) and hippuric acid (32) are probably formed in this way. Such a mechanism is also a plausible one for the synthesis of true peptide bonds between amino acids.

SUMMARY

Purified extracts of acetone-dried pigeon liver catalyze a stoichiometric reaction between adenosine triphosphate, glutamate, and ammonia, in the presence of Mg^{++} ions, to form glutamine, adenosine diphosphate, and inorganic phosphate. The preparation and some of the properties of the enzyme are described. The stoichiometry of the reaction is shown by measurements of amide formation, ammonia utilization, inorganic phosphate liberation, and H^+ ion formation.

In this system other carboxylic acids than glutamic acid do not form amides. Hydroxylamine, hydrazine, and methylamine can be substituted for ammonia; a hydroxamic acid is formed when hydroxylamine is present. Adenosine monophosphate and adenosine diphosphate cannot replace adenosine triphosphate in amide synthesis. Mn^{++} ions can be substituted for Mg^{++} ions. Reducing agents such as cyanide or sulfhydryl compounds must be added to obtain maximum rates of reaction.

Synthesis of glutamine is strongly depressed by low concentrations of sodium fluoride. High concentrations of methionine sulfoxide inhibit competitively with respect to glutamate.

Similar enzyme activity is found in acetone powder extracts of brain, liver, and kidney of a number of vertebrates.

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